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Deletion of the G2A receptor fails to attenuate experimental autoimmune encephalomyelitis

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Abstract

Lysophosphatidylcholine (LPC) is a chemotactic lysolipid produced during inflammation by the hydrolytic action of phospholipase A₂ enzymes. LPC stimulates chemotaxis of T cells *in vitro* through activation of the G protein-coupled receptor, G2A. This has led to the proposition that G2A contributes to the recruitment of T cells to sites of inflammation and thus promotes chronic inflammatory autoimmune diseases associated with the generation and subsequent tissue infiltration of auto-antigen-specific effector T cells. However, one study suggests that G2A may negatively regulate T cell proliferative responses to antigen receptor engagement and thereby attenuates autoimmunity by reducing the generation of autoreactive T cells. To address the relative contribution of these G2A-mediated effects to the pathophysiology of T cell-mediated autoimmune disease, we examined the impact of G2A inactivation on the onset and severity of murine experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (MS). Wild type (G2A^{+/+}) and G2A-deficient (G2A^{-/-}) C57BL/6J mice exhibited a similar incidence and onset of disease following immunization with MOG₃₅₋₅₅ peptide. Disease severity was only moderately reduced in G2A^{-/-} mice. Similar numbers of MOG₃₅₋₅₅ specific T cells were generated in secondary lymphoid organs of MOG₃₅₋₅₅-immunized G2A^{+/+} and G2A^{-/-} mice. Comparable numbers of T cells were detected in spinal cords of G2A^{+/+} and G2A^{-/-} mice. We conclude that the proposed anti-proliferative and chemotactic functions of G2A are not manifested *in vivo* and therefore therapeutic targeting of G2A is unlikely to be beneficial in the treatment of MS.

Keywords

Multiple sclerosis; G2A; Lysophosphatidylcholine; T lymphocyte; Chemotaxis

1. Introduction

Regulatory pathways controlling the ability of T cells to migrate into sites of local inflammation are potential candidates for therapeutic inhibition in organ-specific autoimmune diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA). The migratory characteristics of different lymphocytes are conferred by their respective repertoire of surface-expressed chemotactic receptors (Ebert et al., 2005). In addition to the large family of classical chemokines and chemokine receptors, pro-inflammatory lysolipids have gained recognition as important mediators of inflammatory cell migration through the activation of specific G

protein-coupled receptors (GPCRs) (Graler and Goetzl, 2002). The local generation of lysolipids in inflamed tissue is mediated primarily by the hydrolytic action of secretory phospholipase A₂ (sPLA₂) enzymes on membrane and lipoprotein phospholipids (Pruzanski et al., 2005; Touqui and Alaoui-El-Azher, 2001). Lysophosphatidylcholine (LPC) is a major proinflammatory lysolipid product of sPLA₂ (Kougiass et al., 2006)₂ and is also liberated from cells undergoing apoptosis (Kim et al., 2002). Both these processes can contribute to the increases in local concentrations of LPC in inflamed tissue.

LPC stimulates T cell chemotaxis through the activation of G2A (Radu et al., 2004), a GPCR expressed on all major hemato-lymphoid cell-types (Parks et al., 2005). It has therefore been proposed that G2A activation by proinflammatory lysolipids plays a role in the recruitment of T cells into inflammatory lesions and thereby promotes the onset and/or increases the severity of T cell-mediated inflammatory autoimmune diseases such as MS. In support of this notion, PLA₂ activity was shown to be high in MS lesions and its inhibition led to a significant reduction in the onset and progression of experimental autoimmune encephalomyelitis (EAE) in mice (Kalyvas and David, 2004; Pinto et al., 2003). However, while the lysolipid-sensitive chemotactic action of G2A would be expected to exacerbate EAE by promoting T cell infiltration, the results of one previous study suggest that G2A may actually reduce the susceptibility of mice to autoimmunity (Le et al., 2001). This study compared G2A-deficient (G2A^{-/-}) and G2A-sufficient (G2A^{+/+}) mice on mixed BALBc/129Sv genetic backgrounds and revealed an increased susceptibility of the former to a late-onset (>1 year old) systemic autoimmune syndrome with features of human systemic lupus erythematosus (SLE) (Le et al., 2001). In the same study, T cells from G2A^{-/-} mice were found to exhibit hyperproliferative responses to anti-CD3 antibody-mediated antigen receptor crosslinking *in vitro* (Le et al., 2001). The authors of this study concluded that G2A may negatively regulate the proliferative response of T cells to auto-antigens and that mice lacking this receptor are therefore predisposed to the development of autoimmunity due to uncontrolled autoreactive T cell expansion. However, there is no published study demonstrating an effect of G2A deficiency on antigen-driven T cell expansion *in vivo*. Furthermore, examination of the consequences of G2A deficiency in a pure inbred mouse strain commonly used in studies of autoimmunity has not been reported and is required to overcome any potentially confounding effects of variations in genetic background.

The cumulative data suggest that G2A could modulate one or more immunoregulatory processes in T cells governing the susceptibility to MS. The chemotactic action of G2A may exacerbate MS by promoting the recruitment of auto-reactive T cells into the CNS, while its proposed anti-proliferative action may actually limit the production of these auto-reactive effector T cells in secondary lymphoid organs to attenuate disease. To investigate the relative contribution of these G2A-mediated activities to the pathophysiology of MS, we examined the impact of G2A deficiency on the development and progression of EAE. Genetically matched C57BL/6J G2A^{+/+} and C57BL/6J G2A^{-/-} mice were immunized with myelin oligodendrocyte glycoprotein peptide (MOG₃₅₋₅₅) and the kinetics and severity of EAE were compared. We measured the production of auto-reactive (MOG₃₅₋₅₅-specific) T cells in secondary lymphoid organs of immunized mice to address anti-proliferative effects of G2A. We also examined the frequency of T cells in spinal cords to address the potential chemotactic action of G2A on T cell recruitment into the CNS. Disease severity was only moderately reduced in G2A^{-/-} mice. Comparable numbers of MOG₃₅₋₅₅-specific effector T cells were generated in the secondary lymphoid organs of G2A^{+/+} and G2A^{-/-} mice, and similar numbers of T cells were present in their spinal cords. Thus, there is no evidence supporting an anti-proliferative role for G2A in T cells capable of attenuating the expansion of autoreactive T cells *in vivo* and modulating EAE susceptibility. Furthermore, similar numbers of T cells in the CNS of G2A^{+/+} and G2A^{-/-} mice suggest that G2A-mediated chemotactic action is not penetrant *in vivo* and does not influence the pathogenesis of EAE.

2. Materials and methods

2.1. Mice

Wild type (G2A^{+/+}) and G2A^{-/-} mice were backcrossed 12 generations onto the C57BL/6J background were derived by inter-crossing N12 C57BL/6J heterozygotes (G2A^{+/-}).

2.2. EAE induction and evaluation

Eight week old G2A^{+/+} and G2A^{-/-} mice were immunized subcutaneously with 150 µg of myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 (Biosynthesis, Lewisville, TX) emulsified with 50 µg of *Mycobacterium tuberculosis* in incomplete Freund's adjuvant as previously described (Adams et al., 2007). Mice were intraperitoneally injected with pertussis toxin (500 ng) at the time of immunization and 2 days later. Clinical signs of EAE were assessed daily for 30 days using a standard scale of 0-6 as follows: 0, no clinical signs; 1, loss of tail tone; 2, flacid tail; 3, incomplete paralysis of one or both hind legs; 4, complete hind limb paralysis; 5, moribund requiring euthanization; 6, death. For each group of mice, a Cumulative Disease Index (CDI) was calculated based on the sum of the daily averaged clinical scores. All mouse studies were performed with the approval of the University of Alabama institutional animal care and use committee.

2.3. CFSE labeled T cell proliferation assay

Peripheral lymph node cells (10⁷) from wild type or G2A^{-/-} mice were labeled with 2.5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) and subsequently cultured in the presence or absence of plate-bound anti-CD3 antibody (100 ng) in 1 ml RPMI medium containing 10% FCS. Five days later, cells were stained with phycoerythrin (PE)-conjugated anti-CD4 antibody, analyzed using a FACSCalibur, and data analyzed using CellQuest software (BD Biosciences, San Jose, CA).

2.4. Flow cytometric analysis of secondary lymphoid organs

Spleens and peripheral lymph nodes (inguinal, iliac, mediastinal, axillary) were harvested from G2A^{+/+} and G2A^{-/-} mice 15 days following MOG immunization. Tissues were teased in PBS containing collagenase D (100 µg/ml) and subsequently passed through a 45 µm cell strainer. Single cell suspensions were stained with the following combinations of antibodies (BD Pharmingen): CD4^{PERCP}, CD62L^{PE} and CD44^{APC}. For quantification of MOG-specific T cells in secondary lymphoid organs, 2×10⁶ spleen cells from C57BL/6J Ly5.1 mice were incubated (in 200 µl RPMI containing 10% FCS) with 2×10⁶ lymph node or spleen cells from G2A^{+/+} Ly5.2 or G2A^{-/-} Ly5.2 mice immunized 14 days previously with MOG₃₅₋₅₅ peptide to induce EAE in the presence or absence of 1 µg MOG₃₅₋₅₅ peptide for 24 h. Brefeldin was added to cultures for the last 6 h of culture and interferon-γ-producing T cells were measured by intracellular staining with an APC-conjugated anti-interferon-γ specific antibody (BD Pharmingen) following the manufacturers protocol (BD Cytotfix/Cytoperm Plus, BD Biosciences). FITC-conjugated anti-Ly5.1 and PE-conjugated anti-Ly5.2 antibodies were used together with PERCP-conjugated anti-CD4 and APC-conjugated anti-interferon-γ antibodies to measure exclusively the numbers of interferon-γ producing CD4⁺ T cells from Ly5.2 G2A^{+/+} and Ly5.2 G2A^{-/-} mice. Cells were analyzed using a FACSCalibur and data analyzed using CellQuest software (BD Biosciences, San Jose, CA).

2.5. Flow cytometric analysis of spinal cords

Spinal cords were removed from G2A^{+/+} and G2A^{-/-} mice with active EAE (day 15 following MOG₃₅₋₅₅ immunization) after perfusion with PBS. Spinal cords were passed through a cell strainer, washed in PBS, resuspended in 40% Percoll, and subsequently layered onto 70% Percoll. After centrifugation at 2000 rpm for 25 min, cells were removed from the interface

and washed in PBS. Cells were incubated with anti-CD16/32 (24G2, FcR block) and subsequently stained with anti-CD4^{Fluorescein} (GK1_{CR1}.5), antiCD-8^{PE} (53-6.7) and anti-CD45^{APC} (30F11) antibodies (eBiosciences, San Diego, CA). Cells were analyzed using a FACSCalibur and data analyzed using CellQuest software (BD Biosciences, San Jose, CA).

2.6. Statistical analysis

Statistical significance between G2A^{+/+} and G2A^{-/-} mice was calculated using the Wilcoxon signed rank test using GraphPad Software (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Deletion of G2A mildly reduces the severity of MOG₃₅₋₅₅-induced EAE in C57BL/6J mice

To determine whether the previously postulated chemotactic (Radu et al., 2004) or anti-proliferative (Le et al., 2001) functions of G2A play a role in the pathogenesis of EAE, we immunized G2A^{+/+} and G2A^{-/-} mice with MOG₃₅₋₅₅ peptide and compared the kinetics and severity of disease in the two groups of animals. The incidence of disease was identical in G2A^{+/+} and G2A^{-/-} mice (100%) and disease onset was similar in the two groups of mice (14.7 days vs 13.8 days). The overall course of disease was not significantly different between G2A^{+/+} and G2A^{-/-} mice, despite a mild reduction in disease severity in G2A^{-/-} mice compared to their G2A^{+/+} counterparts at later time-points (G2A^{-/-}: CDI 41 vs G2A^{+/+}:50; CDI=Cumulative Disease Index) (Table 1 and Fig. 1).

3.2. G2A deficiency does not alter the frequency of infiltrating T cells in the spinal cord

As in MS, EAE is a T cell-driven autoimmune disease in which both the generation and subsequent CNS infiltration of autoreactive T cells are central to its pathogenesis. In this regard, it has been suggested that the proposed anti-proliferative action of G2A could limit the production of autoreactive effector T cells to attenuate EAE, while its chemotactic action could exacerbate EAE by promoting the recruitment of autoreactive T cells into the CNS. The absence of a significant effect of G2A deficiency on the course of EAE (Table 1 and Fig. 1) could therefore be explained by the co-penetrance of these two opposing actions; in other words, an increase in the proliferative expansion of autoreactive (MOG₃₅₋₅₅-specific) effector T cells in secondary lymphoid organs of G2A^{-/-} mice may have been counter-balanced by a subsequent reduction in their recruitment into the CNS so that clinical symptoms of EAE were not significantly affected. To address this possibility, we first measured the number of T cells in spinal cords of G2A^{+/+} and G2A^{-/-} mice with EAE by flow cytometry (15 days post-MOG₃₅₋₅₅ immunization). The number of CD4 and CD8⁺ T cells in the spinal cords of G2A^{+/+} and G2A^{-/-} mice were comparable (Fig. 2).

3.3. G2A^{-/-} mice generate normal numbers of MOG₃₅₋₅₅-specific T cells

G2A deficiency on a mixed BALBc/sv129 genetic background was reported to confer susceptibility to a late-onset (>1 year old) spontaneous systemic autoimmune syndrome similar to SLE (Le et al., 2001). Hyperproliferative responses of T cells from these G2A^{-/-} mice to antibody-mediated antigen receptor crosslinking *in vitro* (Le et al., 2001) led to the proposition that uncontrolled expansion of autoreactive T cells in the absence of G2A leads to breakdown of immune tolerance and autoimmunity. However, we were unable to detect any signs of this SLElike syndrome (peripheral blood, lymph node and spleen lymphocyte numbers, hyperglobulinemia) caused by G2A deficiency in comparisons of genetically matched ageing G2A^{+/+} and G2A^{-/-} mice on a C57BL/6J (N10) background (Parks et al., 2006) (B.W.P., J.H.S.K., unpublished data). Nevertheless, we did observe that T cells from C57BL/6J G2A^{-/-} mice underwent a significantly greater number of cell divisions following stimulation with antigen receptor-crosslinking anti-CD3 antibodies compared to their G2A^{+/+} counterparts (Fig.

3), confirming the reported *in vitro* hyperproliferative responsiveness of G2A^{-/-} T cells (11). In the absence of any published study examining the impact of G2A on antigen-induced T cell proliferative expansion *in vivo*, it is possible that this hyperproliferative effect of G2A deficiency may only be manifested in T cells artificially stimulated *in vitro*. To address this issue, we first broadly examined leukocyte numbers in the spleen and lymph nodes of MOG₃₅₋₅₅-immunized G2A^{+/+} and G2A^{-/-} mice (15 days following MOG₃₅₋₅₅ immunization) by flow cytometry. Spleen and lymph nodes from G2A^{+/+} and G2A^{-/-} mice contained equivalent numbers of leukocytes (data not shown). We observed no significant differences in the relative numbers of CD4⁺ and CD8⁺ T cells, CD19⁺ B cells and CD11b⁺ myeloid cells in the spleen or lymph nodes of G2A^{+/+} and G2A^{-/-} mice 15 days following MOG₃₅₋₅₅ immunization (Fig. 4A-B). We also measured the frequency of total effector/memory CD4⁺ T cells (CD44^{high}CD62L^{low} CD4⁺ T cells) in secondary lymphoid organs of MOG₃₅₋₅₅-immunized G2A^{+/+} and G2A^{-/-} mice and no significant differences were detected (Fig. 5A). Finally, we examined the frequency of auto-antigen-specific T cells generated in G2A^{+/+} and G2A^{-/-} mice (15 days following MOG₃₅₋₅₅ immunization) by measuring the numbers of T cells capable of responding (by induction of interferon- γ production) to MOG₃₅₋₅₅ peptide stimulation *in vitro*. We cultured spleen or lymph node cells from MOG₃₅₋₅₅-immunized mice (Ly5.2) with antigen presenting cells (spleen cells from genetically-matched Ly5.1 C57BL/6J mice) in the presence or absence of MOG₃₅₋₅₅ peptide. The number of Ly5.2 CD4⁺ T cells capable of producing interferon- γ in response to MOG₃₅₋₅₅ stimulation *in vitro* was comparable in the spleens and lymph nodes of G2A^{+/+} and G2A^{-/-} mice (% MOG₃₅₋₅₅-specific T cells in peripheral lymph node CD4⁺ population: G2A^{+/+} 0.78 $\pm$ 0.45%, G2A^{-/-} 0.71 $\pm$ 0.43%) (Fig. 5B). Similar results were obtained for MOG₃₅₋₅₅-specific CD8⁺ T cells, although they were present at significantly lower numbers in G2A^{+/+} and G2A^{-/-} mice compared to their CD4⁺ counterparts (data not shown).

4. Discussion

The cumulative data suggest that G2A could modulate the migratory and/or proliferative properties of T cells to influence the pathogenesis of EAE (Le et al., 2001; Radu et al., 2004). The present study addresses the relative contribution of these effects by demonstrating that G2A deficiency has no significant influence on the expansion of autoreactive T cells induced by MOG₃₅₋₅₅ immunization (Fig. 5) and does not result in a reduction in the number of infiltrating T cells in the CNS (Fig. 2).

G2A^{-/-} mice were originally reported to exhibit an increased susceptibility to a late-onset SLE-like syndrome (Le et al., 2001). Based on the hyperproliferative responses of G2A^{-/-} T cells to anti-CD3-mediated TCR crosslinking *in vitro*, it was proposed that this SLE-like syndrome developed as a result of deregulated proliferative expansion of autoreactive T cell clones (Le et al., 2001). However, mice used in that study were of a mixed genetic background (BALBc/129Sv) and so the susceptibility of G2A^{-/-} mice to this SLE-like syndrome may have been conferred primarily by differences in uncharacterized genetic loci. Nevertheless, we did confirm that T cells from N12 C57BL/6J G2A^{-/-} mice undergo a hyperproliferative response to anti-CD3-mediated TCR crosslinking *in vitro* compared to those from genetically matched N12 C57BL/6J G2A^{+/+} mice (Fig. 3). We reasoned, therefore, that in accordance with the mechanism postulated to explain the SLE-like syndrome in G2A^{-/-} mice (Le et al., 2001), hyperproliferative responses of T cells to MOG₃₅₋₅₅ antigen in our G2A^{-/-} animals could promote EAE. However, this was not the case (Fig. 1 and Table 1) and G2A deficiency had no significant effect on autoreactive (MOG₃₅₋₅₅-specific) T cell expansion *in vivo* (Fig. 5). One possible explanation for this discrepancy between *in vitro* and *in vivo* effects of G2A deficiency with respect to T cell proliferative expansion may be the artificial manner by which T cells were stimulated *in vitro*. For example, recapitulation of T cell activation by antigen-MHCII/TCR engagement, as opposed to anti-CD3-mediated TCR crosslinking, may not elicit

hyperproliferative responses of G2A^{-/-} T cells. However, no study reporting the impact of G2A deficiency on *in vitro* and *in vivo* T cell responses in the context of an antigen-specific TCR transgenic model has been reported, yet is clearly warranted. Nevertheless, our data clearly demonstrate that, at least in the pathophysiological context of EAE, G2A deficiency does not potentiate autoreactive T cell expansion *in vivo* and thus fails to promote autoimmunity. Instead, a mild reduction in EAE severity was observed amongst G2A^{-/-} mice (Fig.1 and Table 1) compared to their G2A^{+/+} counterparts. We therefore conclude that autoreactive T cells are generated and subsequently infiltrate the CNS at comparable levels in G2A^{+/+} and G2A^{-/-} mice to propagate neuroinflammation and thereby precipitate severe symptoms of EAE. It is therefore unlikely that inhibition of G2A would provide significant clinical benefit in MS patients.

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Fig. 1.

The clinical course of active EAE is similar in G2A^{-/-} mice compared to G2A^{+/+} mice. Active EAE was induced with MOG₃₅₋₅₅ peptide and symptoms scored for 30 days as described in Materials and methods. Results shown are the daily mean clinical score for G2A^{+/+} ($n=14$) and G2A^{-/-} mice ($n=15$).

Fig. 2.

Comparable CNS T cell infiltration in G2A^{+/+} and G2A^{-/-} mice. Cellular composition of spinal cords from G2A^{+/+} and G2A^{-/-} mice 15 days following EAE induction (MOG₃₅₋₅₅ immunization). (A) Numbers of CD4⁺ and CD8⁺ T cells in spinal cords from G2A^{+/+} and G2A^{-/-} mice. (B) Representative flow cytometric plots of spinal cord T cell infiltrates from G2A^{+/+} and G2A^{-/-} mice.

Fig. 3.

Increased proliferation of G2A^{-/-} T cells in response to antigen receptor crosslinking *in vitro*.

(A) Representative flow cytometric plots showing diminution of CFSE fluorescence in dividing CD4⁺ T cells from G2A^{+/+} and G2A^{-/-} mice stimulated with anti-CD3 antibody (100 ng/ml) for 5 days. Note the increased numbers of G2A^{-/-} CD4⁺ T cells with low CFSE fluorescence, indicating they have undergone more cell divisions compared to their G2A^{+/+} counterparts.

(B) Graph showing the proportion of CD4⁺ T cells having undergone the indicated number of cells divisions. Data shown are representative of 3 independent experiments.

Fig. 4. Cellular composition of spleen (A) and peripheral lymph nodes (B) of MOG₃₅₋₅₅-immunized G2A^{+/+} and G2A^{-/-} mice (15 days following immunization). Shown are the relative distribution of major hematolymphoid cell-types and are the mean±standard deviation of data obtained from 6 G2A^{+/+} and 6 G2A^{-/-} mice.

Fig. 5.

Effector T cells and MOG₃₅₋₅₅-specific T cells are present at comparable frequencies in the secondary lymphoid organs of MOG₃₅₋₅₅-immunized G2A^{+/+} and G2A^{-/-} mice. (A) Flow cytometric analysis of effector/memory CD4⁺ T cells (CD4⁺CD44^{high}CD62L^{low}) in the spleen and peripheral lymph nodes of MOG₃₅₋₅₅-immunized G2AR^{+/+} and G2A^{-/-} mice (15 days following immunization). The percentage of cells present within each cell population is indicated on each flow cytometric plot. Graph with the percentage of effector/memory cells within the CD4⁺ cell population is shown alongside. Data shown are the average from analysis of 4 G2A^{+/+} and 4 G2A^{-/-} mice. (B) Flow cytometric analysis of interferon- γ (IFN- γ)-producing CD4⁺ T cells in the spleen and peripheral lymph nodes of MOG₃₅₋₅₅-immunized G2A^{+/+} and G2A^{-/-} mice (15 days following immunization). Representative flow cytometric plots showing the percentage of CD4⁺ T cells stimulated to produce IFN- γ when cultured with antigen presenting cells in the absence of MOG₃₅₋₅₅ peptide (upper panel) or the presence of MOG₃₅₋₅₅ peptide (lower panel). Graphs showing the percentage of IFN γ -producing cells within the CD4⁺ population, and the actual percentages of MOG₃₅₋₅₅-specific IFN- γ -producing cells within the CD4⁺ population (% with MOG₃₅₋₅₅ - % without MOG₃₅₋₅₅), are shown alongside. Data shown are the average from analysis of 6 G2A^{+/+} and 6 G2A^{-/-} mice.

Table 1EAE in G2A^{+/+} mice and G2A^{-/-} mice

	CDI ^a	Disease onset ^b	Disease incidence ^c	Mortality
G2A ^{+/+} n=14	50	14.7 days	100	None
G2A ^{-/-} n=15	41	13.8 days	100	None

^a Cumulative Disease Index is the mean of the sum of daily clinical scores observed between 7 and 30 days post-MOG₃₅₋₅₅ immunization.

^b Disease onset is defined as the first of two consecutive days in which all mice exhibited a clinical score of two or more.

^c Disease incidence is defined as the percent of mice that displayed any clinical signs of disease.