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Journal of Neuroimmunology 191 (2007) 8 – 15

Journal of
Neuroimmunology

www.elsevier.com/locate/jneuroim

Review article

Understanding the behavior of invariant NKT cells in autoimmune diseases

Takashi Yamamura [a,*], Kaori Sakuishi [a], Zsolt Illés [b], Sachiko Miyake [a]

[a] *Department of Immunology, National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan*
[b] *Department of Neurology, University of Pecs, Hungary*

Received 28 August 2007; accepted 7 September 2007

Abstract

Invariant NKT (iNKT) cells are a unique subset of lymphocytes that recognize glycolipid antigens presented by a monomorphic glycoprotein CD1d. Numerous works have shown that iNKT cells may serve as regulatory cells in autoimmune diseases including multiple sclerosis (MS). However, recent studies have revealed that the presence of iNKT cells accelerates some inflammatory conditions, implying that their protective role against autoimmunity is not predetermined. Here we review recent information concerning the mechanism of how iNKT cells intervene or promote autoimmune inflammation. Although iNKT cells are thought to be specific for a limited set of glycolipids, they may cross-react to self and non-self ligands. Regarding the response to non-self, it is now known that iNKT cells produce enormous amounts of proinflammatory cytokines during the course of infectious diseases, which is triggered by TCR ligation by microbial lipids, cytokines produced from APCs or both. Whereas the strongly activated iNKT cells play a beneficial role in combating environmental pathogens, they could play a deleterious role in autoimmunity by producing disease-promoting cytokines. However, iNKT cells in the steady state would retain an ability to produce anti-inflammatory cytokines, which is needed for terminating the ongoing inflammation. Though an initial trigger for their regulatory responses remains elusive, our recent work indicates that iNKT cells may start regulating inflammation after sensing the presence of IL-2 in addition to recognizing a ubiquitous endogenous ligand. Understanding of how iNKT cells regulate autoimmunity should lead to a more sophisticated strategy for controlling autoimmune diseases.

Keywords: NKT cells; iNKT cells; Multiple sclerosis

Contents

* Corresponding author. Tel.: +81 42 346 1723; fax: +81 42 346 1753.
E-mail address: yamamura@ncnp.go.jp (T. Yamamura).

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doi:10.1016/j.jneuroim.2007.09.014

1. Introduction

Invariant NKT (iNKT) cells are regulatory T lymphocytes reactive to lipid antigen presented by a monomorphic glycoprotein CD1d (Bendelac et al., 2007; Kronenberg, 2005; Taniguchi et al., 2003). Many previous reports have documented that the number or function of iNKT cells is altered in patients with autoimmune diseases such as multiple sclerosis (MS) (Araki et al., 2003; Illés et al., 2000; Kojo et al., 2001; van der Vliet et al., 2001b; Wilson et al., 1998). Studies using transgenic mice that over- or under-express iNKT cells have basically supported the involvement of iNKT cells in the pathogenesis of autoimmune diseases (Miyake and Yamamura, 2007a). Moreover, stimulating iNKT cells with synthetic glycolipids has proven effective for preventing experimental autoimmune encephalomyelitis (EAE) (Miyamoto et al., 2001; Pál et al., 2001) or spontaneous type 1 diabetes (T1D) in NOD mice (Naumov et al., 2001; Sharif et al., 2001), indicating the important role of iNKT cells in controlling pathogenic autoreactivity and maintaining immune homeostasis (Miyake and Yamamura, 2007b). However, more recent studies have shown that iNKT cells may augment inflammatory conditions in models of arthritis (Chiba et al., 2005; Kim et al., 2005; Ohnishi et al., 2005), $CD8^+$ T cell-mediated diabetes (Griseri et al., 2005), experimental colitis (Ronet et al., 2005; Ueno et al., 2005) and airway hypersensitivity reactions (Akbari et al., 2003; Meyer et al., 2007). These results indicate that unlike $CD4^+$ $CD25^+$ regulatory T cells that appear to be a faithful regulator of unwanted immune responses (Sakaguchi and Sakaguchi, 2005), iNKT cells' help is only conditional and would occasionally take part in augmentation of harmful inflammation. How activation of iNKT cells manifests such opposing results and what is an initial trigger for the regulatory iNKT cell responses has remained to be unanswered. Here we review recent advances in the research of iNKT cells that may be relevant for understanding the "Janus-like" behavior of iNKT cells (Wilson and Delovitch, 2003). Our ultimate goal is to seek ways for making iNKT cells serve as a reliable guardian for our health.

2. General properties of iNKT cells

Although iNKT cells express T cell receptor (TCR) α- and β-chains, their TCR diversity is very limited owing to their expression of a single α-chain (Vα14-Jα18 in mice, Vα24-Jα18 in human) coupled with a β-chain rearranged with a limited Vβ gene segments (Vβ8.2, Vβ2 and Vβ7 in mice, Vβ11 in human). Unlike conventional T cells, they constitutively express memory/activated T cell phenotype and are capable of producing enormous amounts of pro- and anti-inflammatory cytokines shortly after TCR engagement (Bendelac et al., 2007; Kronenberg, 2005; Miyake and Yamamura, 2005; Taniguchi et al., 2003). The cytokine burst following iNKT cell activation then triggers a maturation process in downstream cells such as NK cells, dendritic cells (DCs), B cells and T cells, leading to subsequent alteration of a broad range of adaptive immune responses. It is widely accepted that they could behave very much like innate lymphocytes rather than conventional T cells (Mempel et al., 2002), and owing to the rapidity with which they respond to various stimuli, they play an important role in bridging innate and adaptive arms of immune response.

The ability of iNKT cells to produce regulatory cytokines is so outstanding that they could efficiently alter an adaptive immune response. Mouse iNKT cells can produce interferon-γ (IFN- γ), IL-2 (Jiang et al., 2005), -3 (Leite-de-Moraes et al., 2002), -4, -5, -13, -17, -21 (Coquet et al., 2007), GM-CSF (Leite-de-Moraes et al., 2002), and osteopontin (Diao et al., 2004) after an optimal engagement of TCR. However, it does not mean that iNKT cells would purposefully use all the listed cytokines. In fact, it can be assumed that except for extreme conditions (like stimulation with strong agonists), iNKT cells may produce only a set of Th1 or Th2 cytokines in physiological conditions. We support this postulate because the TCR engagement by an endogenous ligand is likely to be modest or suboptimal in most situations (Sakuishi et al., 2007). With regard to their role in balancing immune homeostasis, an organized production of Th1, Th2 or Th17 cytokines is probably required for iNKT cells to conduct meaningful jobs.

3. Exogenous glycolipids stimulatory for iNKT cells

Since a marine sponge-derived glycosphingolipid, α-galactosylceramide (α-GalCer), was discovered as a potent ligand for iNKT cells (Kawano et al., 1997), a synthetic α-GalCer has widely been used for study of iNKT cells as a surrogate ligand (Fig. 1). It is now established that two lipid chains of α-GalCer are inserted to hydrophobic grooves of the CD1d glycoprotein expressed by antigen presenting cells (APCs) (McCarthy et al., 2007), whereas the α-linked sugar moiety is accessible and recognized by the TCR of iNKT cells. Recently, the crystal structure of the invariant TCR and CD1d loaded with α-GalCer has shown a very unique orientation of TCR towards CD1d (Borg et al., 2007), which allows a selective involvement of the invariant α-chain for recognition of the α-linked sugar.

Comparison of α-GalCer with its structurally altered analogues has provided important insights into how iNKT cells may differentially respond to glycosphoingolipids with lipid tail variants (Brutkiewicz, 2006; Miyake and Yamamura, 2007b). As a representative example, we showed previously that an α-GalCer analogue called OCH (Miyamoto et al., 2001; Oki et al., 2004, 2005), with a shorter sphingosine chain (Fig. 1), would selectively stimulate IL-4 production from iNKT cells, whereas α-GalCer stimulation induces both IL-4 and IFN-γ. Accordingly, OCH stimulation of iNKT cells favors a Th2 bias of immune responses *in vivo*, as compared to α-GalCer stimulation.

α-linked sugars such as α-GalCer are not recognized as a product of mammalian cells, implying that α-GalCer is not a physiological ligand for iNKT cells. Currently, it is well recognized that iNKT cells can be activated during infectious diseases (Tupin et al., 2007). Interestingly, it has been reported that α-GalCer-like glycosphingolipids are rather ubiquitously found in the environment, indicating that α-GalCer may be actually derived from bacteria residing with the marine sponge. Whether or not α-GalCer is derived from bacteria, we may ask a number of questions as to whether infectious diseases may influence on autoimmune disease via activation of iNKT cells

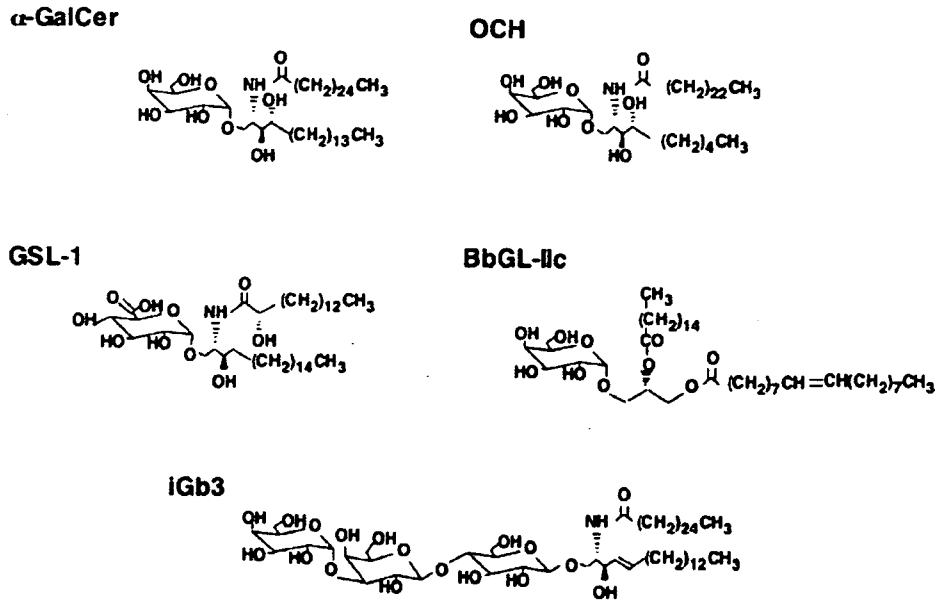

Fig. 1. Structure of glycolipid ligands for iNKT cells. Shown here are the structure of NKT cell agonists: α-galactosylceramide (α-GalCer) (Kawano et al., 1997), an α-GalCer analog called OCH, bearing a shorter sphingosine chain (Miyamoto et al., 2001), *Sphingomonas*-derived glycosphingolipid GSL-1 (Kinjo et al., 2005), *Borrelia burgdorferi*-derived diacylglycerol glycolipid BbGL-IIc (Kinjo et al., 2006), and isoglobotryhexosylceramide (iGb3) (Zhou et al., 2004).

(Godfrey and Berzins, 2006). Although multiple pathways are operative for iNKT cell activation in facing microbial challenge, it has been shown that glycosphingolipids from LPS-negative α-Proteobacteria such as *Sphingomonas* (Fig. 1) could stimulate a proportion of iNKT cells (Kinjo et al., 2005; Mattner et al., 2005). They also found that diacylglycerol glycolipids, extracted from *Borrelia burgdorferi*, stimulate at least 25% of iNKT cells (Kinjo et al., 2006; Kinjo et al., 2005). It is currently thought that arthritis and carditis found in Lyme disease following *B. burgdorferi* infection may be mediated by an autoimmune process. Whether iNKT cells activated by the diacylglycerol lipids may contribute to the pathogenesis of Lyme disease is an interesting question to be addressed. Likewise, an interesting idea is that relapse of MS following infection may be triggered by iNKT cells that are activated in response to microbial stimuli. Of note is that iNKT cells may produce osteopontin, which is reported to trigger relapses of EAE by promoting the survival of activated T cells in the inflammatory site (Hur et al., 2007).

4. Endogenous ligand for iNKT cells: search is not over

Search for an endogenous ligand of iNKT cells has led to the identification of lysosomal glycosphingolipid isoglobotryhexosylcermiade (iGb3) (Fig. 1), a β-linked sugar capable of stimulating iNKT cells as a potential endogenous ligand for mouse and human iNKT cells (Mattner et al., 2005; Zhou et al., 2004). With regard to the role of iGb3 in adaptive immune responses, Mattner et al. reported that Gram-negative, LPS-positive *Salmonella typhimurium* activates NKT cells through the recognition of iGb3, presented by LPS-activated dendritic cells. However, very recent works have cast doubt on the meaning of the iGb3 discovery (Porubsky et al., 2007; Speak et al., 2007). The study by Zhou et al. (2004) indicated that iGb3 presented by CD1d-expressing $CD4^+CD8^+$ thymocytes should be involved in the thymic positive selection of iNKT cells. Porubsky et al. has then generated iGb3 synthetase deficient mice and examined if iNKT cells are really missing in the mice lacking expression of iGb3. They found that the number and function of iNKT cells were as normal as those seen in wild-type mice. Using highly sensitive HPLC assay, Speak et al. sought for the presence of iGb3 in various mouse and human tissues. The only tissue containing iGb3 was the dorsal root ganglion of mice. No iGb3 was detected in any human tissue (Porubsky et al., 2007; Speak et al., 2007). These new findings do not support the idea that iGb3 is central in the selection of iNKT cells and re-opened the search for endogenous ligands for iNKT cells.

With regard to the pathogenesis of MS, it is interesting to know if brain-derived lipids may stimulate iNKT cells. Although such ligands have not been identified yet for iNKT cells, sulfatide derived from the myelin appears to be a ligand for non-invariant NKT cells or type II NKT cells (Godfrey et al., 2004) that bear diverse TCR repertoire although restricted by CD1d glycoprotein (Jahng et al., 2004; Zajonc et al., 2005). This interesting finding leaves room for exploring presence of myelin-derived ligands for iNKT cells that may play a role in the pathogenesis of MS.

5. Human iNKT cells and autoimmune diseases

iNKT cells' recognition of CD1d ligand is well known for its evolutionary conservation across species barriers as indicated by the fact that both mouse and human iNKT cells share a highly homologous CDR3 of TCR α-chain and would cross-recognize α-GalCer (Spada et al., 1998). However, iNKT cells from mouse and human significantly differ in population size in lymphoid organs and peripheral blood (mouse ≫ human). In addition, a clear functional dichotomy for $CD4^+$ and $CD4^-$

populations is found in human (Gumperz et al., 2002; Lee et al., 2002) but not in mouse (Kronenberg and Gapin, 2002). A lower number of the iNKT cells has led to repeated questions about the actual role of iNKT cells in human. However, studies have shown that human iNKT cells show an outstanding ability to proliferate after *in vitro* (van der Vliet et al., 2001a; Yanagisawa et al., 2002) or *in vivo* stimulation with α-GalCer (Chang et al., 2005). Moreover, patients with rare genetic diseases associated with the absence of iNKT cells are reported to suffer from serious viral infections (Levy et al., 2003; Rigaud et al., 2006). These results support a vital role for iNKT cells in maintaining the human health.

The $CD4^+/CD4^-$ dichotomy of human iNKT cells (Gumperz et al., 2002; Lee et al., 2002) is widely appreciated at present. In brief, $CD4^+$ iNKT cells could produce both pro- and anti-inflammatory cytokines after proper stimulation, indicating their ability to balance immune homeostasis. In contrast, $CD4^-$ iNKT cells predominantly produce proinflammatory cytokines such as TNF-α and IFN-γ, but little Th2 cytokines, which is reminiscent of NK cells rather than T cells. A number of studies have addressed the difference between $CD4^+$ and $CD4^-$ iNKT cells in human disease conditions (Araki et al., 2003; Illés et al., 2000; Takahashi et al., 2003). A striking reduction of the total number of iNKT cells in the peripheral blood from remission state MS has been reported from us in previous studies (Araki et al., 2003; Illés et al., 2000). When the $CD4^+$ and $CD4^-$ iNKT cells were analyzed separately, we again noted a remarkable reduction of $CD4^-$ iNKT cells in MS. However, a reduction of $CD4^+$ iNKT cells was only modest. Furthermore, we generated long-term $CD4^+$ iNKT cell lines from MS and healthy subjects and compared their ability to produce IFN-γ and IL-4. We found that the $CD4^+$ NKT cells from subjects with MS produce much more IL-4 than those from healthy subjects, whereas production of IFN-γ was not significantly different. The data collectively support that Th2 biased $CD4^+$ NKT cells may somehow contribute to maintaining the remission state of MS. In contrast, a Th1 bias of iNKT cells has been reported in human type I diabetes (Kent et al., 2005; Wilson et al., 1998). This bias is characterized by the inability to produce IL-4. A similar Th1 bias was also confirmed by using iNKT cell clones derived from draining lymph nodes of affected pancreas from T1D patients (Kent et al., 2005). As such, Th2 bias of iNKT cells during remission of MS seems to be purposeful, whereas the Th1 bias found in T1D could contribute to enhancing pathogenic autoimmunity.

6. iNKT cells regulate autoimmunity in response to exogenous ligands

By using mice lacking CD1d or TCR Jα18 gene that is required for development of iNKT cells, a number of works have proven the role of iNKT cells in self-tolerance and prevention of autoimmunity. Yet, how iNKT cells actually contribute to maintaining self-tolerance remains largely unknown. Earlier works have mainly asked how an exogenous therapeutic ligand such as OCH would modulate autoimmune disease processes. A single injection of OCH protects against development of EAE. However, a simultaneous injection of anti-IL-4 antibody abrogated the preventive effect of OCH. Moreover, disease protective effects of OCH could not be seen in IL-4 knockout mice, indicating that IL-4 produced from iNKT cells is involved in the disease suppression (Miyamoto et al., 2001). Thus, a single NKT cell stimulation with OCH probably inhibits EAE in an Ag-nonspecific mechanism. In contrast, it has been shown by others that repeated injections of α-GalCer would suppress T1D by promoting differentiation and recruitment of tolerogenic DCs in draining lymph nodes (Chen et al., 2005; Gillessen et al., 2003; Naumov et al., 2001). It is possible that presentation of a tissue-specific antigen by tolerogenic DCs may induce Ag-specific regulatory $CD4^+$ T cells secreting IL-10, which accounts for the protection against diabetes.

Without applying an exogenous glycolipid, Lehuen and colleagues have recently shown that iNKT cells could prevent a T cell-transfer model of diabetes by inducing an anergic state of the pathogenic, islet-specific T cells. In contrast to other related works, this suppression did not require Th2 cytokines but was dependent upon direct cell-cell contact (Beaudoin et al., 2002). Subsequent studies showed that the cellular interaction does not involve CD1d recognition by NKT cells (Kent et al., 2005; Novak et al., 2007). Although the mechanism of iNKT cell-mediated regulation in this model remains unclear, it is reminiscent of our work showing that a newly recognized NKT cells (MR1-restricted Vα19 NKT cells) would mediate immune regulation via direct contact with B cells through ICOS-ICOSL interaction independent of TCR recognition (Croxford et al., 2006).

7. Cytokines instruct iNKT cell response towards Th1 or Th2

Although iNKT cells could conduct a tremendous job following stimulation with exogenous ligands or via direct cellular contact, recent studies on the behavior of iNKT cells during *S. typhimurium* infection have highlighted the importance of iNKT cell recognition of an endogenous CD1d ligand in combating against microbial pathogen (Brigl et al., 2003). The work by Brigl et al. showed that iNKT cells would respond to *S. typhimurium* by producing IFN-γ, when co-cultured with DCs. Interestingly, even stimulation with LPS from *S. typhimurium* could similarly induce the IFN-γ production, indicating the involvement of TLRs rather than TCR engagement by bacterial components. Subsequent experiments showed that this IFN-γ production critically required IL-12 that was derived from DCs via TLRs in a MyD88-dependent way. However, IL-12 was not sufficient to cause the iNKT cell production of IFN-γ. It was thought that the production of IFN-γ would require recognition of endogenous CD1d ligand, as anti-CD1d antibodies proved to block the response. Whether or not iGb3 is involved is still not clear, but these results clarified that iNKT cells would exert a decisive effector function (such as a predominant IFN-γ production) when iNKT cells recognize an endogenous ligand in the presence of an exogenous cytokine.

We have recently explored if cytokines other than IL-12 may induce an effector function of iNKT cells. For this aim, human $CD4^+$ iNKT cell clones were stimulated with various cytokines in the presence of DCs. None of the clones co-cultured with

DCs exhibited any noticeable response in the absence of exogenous cytokines. However, 7 out of 27 clones examined produced a large amount of IL-5 and IL-13 when IL-2 was added to the NKT-DC co-cultures. The amount of IL-5 and IL-13 was comparable to that induced with the most potent ligand α-GalCer. However, α-GalCer never induces such a biased response but stimulates production of a broad spectrum of pro- and anti-inflammatory cytokines. Remarkable production of IL-5 and IL-13 but not of other cytokines was also confirmed by conducting DNA microarray analysis. This surprising result raises two points: 1) human $CD4^+$ iNKT cells may comprise functionally distinct populations, including such IL-5/-13 producing clones, and 2) IL-2 may be a critical factor that induces a physiological Th2 response of iNKT cells. Further analysis showed that the production of Th2 cytokines was dependent on the TCR recognition of CD1d ligand. Indeed, addition of anti-CD1d antibody blocked the response, and CD1d lacking APCs could not induce the response. Furthermore, the combination of IL-2 with a weak TCR stimulus by suboptimal concentration of anti-CD3 antibody has reproduced a similar Th2 cytokine production. These results indicate that IL-2 could play a major role in instructing the iNKT cell population to selectively produce Th2 cytokines (Sakuishi et al., 2007). Taking all these into consideration, we propose that sensing the presence of cytokines is probably one of the most fundamental abilities for the iNKT cells that are to be given only a weak TCR signal *in vivo*.

IL-12 induced production of IFN-γ (Brigl et al., 2003; Mattner et al., 2005) as well as IL-2 induced production of IL-5 (Sakuishi et al., 2007) depends upon the recognition of endogenous ligand via TCR. However, iNKT cells could also produce a large amount of cytokine in response to cytokine signals independently of TCR signals. It has been shown that iNKT cells can be activated by *Escherichia coli* LPS, and produce IFN-γ, but not IL-4. Nagarajan and Kronenberg have shown that the production of IFN-γ was dependent upon LPS-induced IL-12 and IL-18 from APC, but did not require CD1d-mediated presentation of an endogenous Ag. Furthermore, they showed that exposure to a combination of IL-12 and IL-18 sufficiently activated the iNKT cells (Nagarajan and Kronenberg, 2007). TCR-independent production of Th1 cytokine strongly indicates the innate lymphocyte-like property of iNKT cells.

8. Antigen presenting cells for iNKT cells

To evaluate reactivity of iNKT cells, previous works have mostly used dendritic cells (DCs) or unseparated lymphoid cells as APCs. Recently, two groups have used non-professional APCs for stimulating iNKT cells, and obtained interesting results (Bezbradica et al., 2005; Im et al., 2006). The study by Bezradica et al. has compared the ability of DCs, B cells, hepatocytes, and macrophages to present α-GalCer to mouse NKT cells. Whereas presentation with DCs induced a remarkable production of IFN-γ and IL-4 from NKT cells, α-GalCer-loaded hepatocytes or

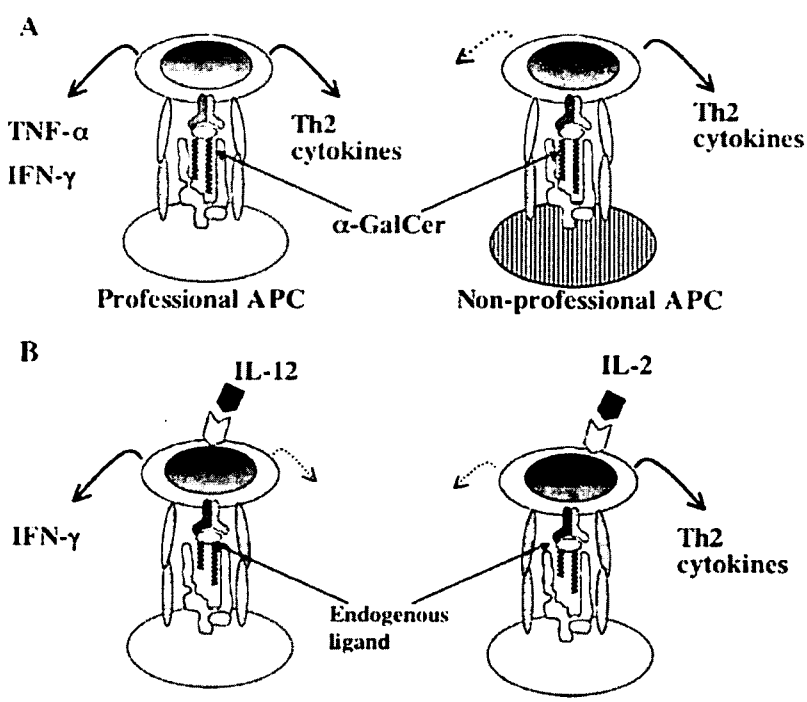

Fig. 2. Activation of iNKT cells by unconventional ways leading to functional bias. A: APC-dependent functional bias of NKT cells. Stimulating NKT cells with α-GalCer presented by professional APCs leads to production of both pro- and anti-inflammatory cytokines (left). However, when non-professional APCs such as Schwann cells (Im et al., 2006) are used, α-GalCer could induce a preferential production of Th2 cytokines from NKT cells. B: Cytokine-dependent functional bias of NKT cells recognizing endogenous ligand/CD1d. NKT cells usually exhibit only a marginal response in response to endogenous ligand bound with CD1d. However, when cytokines are added exogenously, the cells that recognize the endogenous ligand would produce a large amount of selected cytokines. For example, IL-12 induces production of IFN-γ (left) (Brigl et al., 2003), whereas IL-2 provokes IL-5 and IL-13 (right) (Sakuishi et al., 2007).

macrophages did not appear to induce iNKT cells responses. Interestingly, NKT cell stimulation with α-GalCer presented by B cells induced a weak cytokine response characterized by a low production of IL-4. Porcelli and his colleagues have examined the ability of human Schwann cells to present α-GalCer to NKT cells (Im et al., 2006). They showed that iNKT cells produced much lower amounts of proinflammatory cytokines (TNF-α and IFN-γ) but predominantly produced Th2 cytokines (IL-5 and IL-13) when Schwann cells were used as APCs. Although these studies did not examine the NKT cell reactivity to self-CD1d ligand, the results indicate that non-professional APCs tend to provoke production of Th2-associated cytokines from iNKT cells, allowing us to speculate that iNKT cell responses may greatly vary in different organs and tissues resided with different types of APCs.

9. Concluding remarks

Although most previous works have used α-GalCer or anti-CD3 antibody for stimulating iNKT cells to evaluate their functions, recent works have identified various alternative ways by which iNKT cells could be properly and differentially activated (Fig. 2). It is of particular note that iNKT cells exert polarized regulatory functions when exposed to an endogenous CD1d ligand in the presence of cytokines such as IL-12 and IL-2. We speculate that cytokine-triggered activation of iNKT cells should reflect a number of physiological or pathological conditions that could take place in the maintenance of immune homeostasis. Occurrence of Th1 polarization for iNKT cells or robust production of proinflammatory cytokines such as IFN-γ and osteopontin in response to infectious stimuli indicates a new mechanism for exacerbating autoimmune diseases preceded by an infection. Very interestingly, a growing number of potential agonists for iNKT cells have been identified from relatively common pathogens (Tupin et al., 2007). This opens a new possibility that environmental pathogens may play an active role in maintaining the population size and functions of iNKT cells in healthy conditions. Given that the frequency of iNKT cells in the peripheral blood greatly varies among healthy populations, this is an interesting question to be addressed experimentally. This new idea and a prevailing view about the major influence by genetic factors on iNKT cells are not mutually exclusive. Consequently, new approaches exploiting the role of iNKT cells in autoimmunity should probably consider their relation to pathogenic bacteria as well as non-pathogenic microbes.

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Longterm Effect of Intermittent Cyclical Etidronate Therapy on Corticosteroid-Induced Osteoporosis in Japanese Patients with Connective Tissue Disease: 7-Year Followup

SHINJI SATO, TETSUYA TAKADA, YUMIKO KATSUKI, NORIKO KIMURA, YUKO KANEKO, AKIRA SUWA, MICHITO HIRAKATA, and MASATAKA KUWANA

ABSTRACT. ***Objective.*** To determine the efficacy and safety of intermittent cyclical etidronate therapy of up to 7 years for corticosteroid-induced osteoporosis.

Methods. One hundred two Japanese patients who originally participated in a 3-year prospective randomized study were enrolled into an open-label followup study. All patients had received > 7.5 mg of prednisolone daily for at least 90 days before entry into the original study and were randomly assigned to 2 treatment arms: E, those receiving etidronate disodium (200 mg per day) for 2 weeks together with 3.0 g of calcium lactate and 0.75 μg of alphacalcidol daily; and C, controls receiving only the latter. Endpoints included changes from baseline in bone mineral density (BMD) of the lumbar spine and the rate of new vertebral fractures.

Results. The mean (± SD) lumbar spine BMD had increased by 5.9% ± 8.8% ($p = 0.00007$) and 2.2% ± 5.8% ($p = 0.013$) from baseline after 7 years in groups E and C, respectively. This improvement in BMD in group E was significantly better than in group C ($p = 0.02$). The frequency of new vertebral fractures was lower in group E, resulting in reduction of the risk of such new fractures by 67% at year 7 (odds ratio 3.000; 95% confidence interval, 0.604–14.90; $p = 0.18$). There were no severe adverse events in group E during our study.

Conclusion. Our results indicate that longterm (up to 7 years) intermittent cyclical etidronate therapy is safe and effective for prevention and treatment of corticosteroid-induced osteoporosis in patients with connective tissue diseases. (First Release Nov 15 2007; J Rheumatol 2008;35:142–6)

Key Indexing Terms:
CORTICOSTEROID-INDUCED OSTEOPOROSIS
BONE MINERAL DENSITY
BISPHOSPHONATE
CONNECTIVE TISSUE DISEASES

Longterm corticosteroid treatment in patients with connective tissue disease (CTD) causes osteoporosis as the major adverse event. Bisphosphonate therapy has proven to be effective in both prevention and treatment of corticosteroid-induced osteoporosis (CIOP)[1-4]. Guidelines for treating patients with CIOP recommend the use of bisphosphonates as a first-line drug[5]. Nitrogen-containing bisphosphonates, such as alendronate or risedronate, have proven efficacy for both prevention and treatment of CIOP. However, use of these bisphosphonates is associated with gastrointestinal adverse events[6]. We previously conducted a 3-year prospective randomized study to determine the efficacy and safety of etidronate (the first available nitrogen-free bisphosphonate) for treating CIOP[7]. Although longterm followup (7 yrs) of intermittent cyclical etidronate therapy in patients with postmenopausal osteoporosis has been reported[8], few studies are available on the longterm effects of etidronate in patients with CIOP[9]. Further, there are no reports on the continued effectiveness and safety of etidronate for CIOP in patients with CTD. For this reason, we have followed up the original 3-year prospective study for an additional 4 years to determine the longterm efficacy of intermittent cyclical etidronate for treating CIOP in patients with CTD.

From the Department of Internal Medicine, Keio University, School of Medicine, Tokyo; and the Division of Rheumatology, Department of Internal Medicine, Tokai University School of Medicine, Kanagawa, Japan.

S. Sato, MD; T. Takada, MD; Y. Katsuki, MD; N. Kimura, MD; Y. Kaneko, MD; M. Hirakata, MD; M. Kuwana, MD, Department of Internal Medicine, Keio University; A. Suwa, MD, Division of Rheumatology, Department of Internal Medicine, Tokai University School of Medicine.

Address reprint requests to Dr. S. Sato, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: shins@sc.itc.keio.ac.jp

Accepted for publication September 7, 2007.

MATERIALS AND METHODS

Patients. In the original 3-year study, 102 patients with different CTD were enrolled (56 with systemic lupus erythematosus; 12 rheumatoid arthritis; 10 polymyositis/dermatomyositis; 9 vasculitis syndrome; 8 adult-onset Still disease; 5 polymyalgia rheumatica; 1 systemic sclerosis; and 1 Sjögren's syndrome). Patients' ages ranged from 21 to 73 years and they had been taking

> 7.5 mg of prednisolone (PSL) daily for at least 90 days. The results of this study have been reported elsewhere[7].

Study design. In the original 3-year prospective randomized study, all patients were randomly assigned to one of 2 investigational groups. Patients in the etidronate group (E) received 200 mg/day etidronate disodium (Didronel, Sumitomo Pharmaceuticals, Osaka, Japan) for 2 weeks, together with 3.0 g of calcium lactate and 0.75 μg of alphacalcidol (Alfarol, Chugai Pharmaceuticals, Tokyo, Japan) daily for 90 days. This cycle was repeated 28 times during the 7-year observation period. Patients were instructed to take their medication with water at bedtime. The control group (C) received only 3.0 g of calcium lactate and 0.75 μg of alphacalcidol daily for 90 days. In the followup study, the patients were to continue taking the same treatments to which they had been assigned earlier. However, a change of treatment was allowed if their doctor decided that it was necessary for treatment. All patients had provided written informed consent.

Bone mineral density (BMD) and radiological measurements. Lateral and anteroposterior lumbar and thoracic spine radiographs were taken and evaluated at Keio University Hospital at baseline and every year for 7 years. All lumbar and thoracic spine images were evaluated by experienced physicians who were blinded to treatment assignments. The diagnosis of vertebral fracture and osteoporosis was based on the criteria defined by the Japanese Society for Bone and Mineral Research in 1996[10]. A vertebral fracture was defined as: (1) The ratio of the central height (C) to the anterior height (A) of the vertebra was less than 0.8, or the ratio of C to the posterior height (P) was less than 0.8. (2) The ratio of A to P was less than 0.75. (3) A crushed vertebra was recorded when its height was reduced by more than 20% in either A, C, or P compared with the adjacent vertebrae.

Classification of BMD was based on the following criteria: Normal BMD: > 80% of the young adult mean (YAM). Osteopenia: between 70% and 80% of YAM. Osteoporosis: < 70% of YAM.

This definition of osteoporosis (i.e., < 70% of YAM) also corresponds to the osteoporosis criteria recommended by the World Health Organization [less than −2.5 standard deviation (SD) of YAM]. All BMD measurements were made by dual-energy x-ray absorptiometry using an XR-36 (Norland Medical Systems, Fort Atkinson, WI, USA). Because we had already documented changes in bone formation and bone absorption markers in the original 3-year prospective randomized study, we did not monitor biochemical markers of bone turnover in this followup study.

Statistical analysis. The baseline characteristics and homogeneity of the patients' background in an intent-to-treat population was compared between the 2 investigational groups by chi-square test, Student's t-test, and Mann-Whitney U-test, as appropriate. Regardless of whether patients were still receiving the assigned medication, all available BMD data were used to perform an intent-to-treat analysis. If the measurement of the lumbar spine BMD at 7 years was not available, the measurement obtained at the time closest to this was used in the analysis. The patients whose BMD data could not be evaluated correctly because of previous compression fractures are excluded from the analysis. The primary efficacy analysis was based on the differences between the 2 investigational groups in the percentage change of lumbar spine BMD (L2-L4) from baseline to last measurement. The percentage change of BMD from the baseline was compared by an analysis of variance model (SPSS version 14.0). The comparison of percentage change of BMD between the 2 groups was calculated by Student's t-test. Odds ratios adjusted by menopausal status stratum as a factor were calculated for differences of the incidence of vertebral fractures at 7 years between the 2 treatment groups. Significance level was set at 5% and all results expressed as mean ± SD.

RESULTS

At the beginning of this followup study, 7 patients in group E and 6 patients in group C could not be included because of death or loss to followup. There were no significant differences between groups in baseline characteristics in that subset of patients whose data could be used for an intent-to-treat analysis (43 and 45 in groups E and C whose BMD data were available, respectively; Table 1). During 7 years, the average daily dose of PSL in each year was not significantly different between the 2 groups. The number of patients taking steroid in groups E and C was also not significantly different (Table 2). During the followup study, there were no adverse events in either group. However, 2 patients in group E and one in group C died due to progression of their underlying CTD or infection during this study. Ten patients in group C began to receive bisphosphonate as well as alphacalcidol and calcium lactate because their rheumatologist decided that they would benefit from such treatment. On the other hand, only 1 patient in group E was changed from etidronate to alendronate.

After 7 years of treatment, the mean (± SD) percentage change in BMD of the lumbar spine in group E (5.9% ± 8.8%) was significant compared to baseline ($p = 0.00007$). This was also the case, albeit to a lesser extent (2.2% ± 5.8%), in group C ($p = 0.013$; Figure 1). This improvement of BMD was significantly greater in group E than in group C ($p = 0.02$).

In a separate analysis of premenopausal and postmenopausal women, both of these subgroups of group E showed an increase in the mean percentage change in BMD during their treatment course. In premenopausal women, both groups E and C had significant increases of lumbar spine BMD from baseline at 7 years ($p = 0.001$ and $p = 0.02$, respectively). These increases were significantly higher in group E than C in a subgroup of premenopausal women (6.7% ± 9.1% vs 2.3% ± 4.5%; $p = 0.04$). Although the postmenopausal subgroup of group E showed an increase in BMD of the lumbar spine, this failed to achieve significance (2.8% ± 8.0%; $p = 0.23$). BMD of this subgroup in group C remained at baseline (−0.03% ± 7.60%; $p = 0.99$). There were also no significant differences between the 2 groups in this respect.

Analysis of the subgroups based on the baseline BMD revealed that the osteoporosis + osteopenia subgroup in group E showed a significant increase of the lumbar spine BMD from baseline at 7 years ($p = 0.0009$). This increase was significantly greater in group E than in group C at 7 years (7.8% ± 9.5% vs 2.0% ± 6.3%; $p = 0.04$). Both E and C groups of the normal BMD subgroup showed significant increases in lumbar spine BMD at year 7 (3.9% ± 7.7%, $p = 0.03$ and 2.4% ± 5.6%, $p = 0.03$, respectively). Again, there were no significant differences between the 2 groups ($p = 0.40$).

The mean percentage change in lumbar spine BMD in group E improved from baseline by approximately 5% over the 7 years (Figure 2). Although group C showed no decrease of BMD from baseline, the increase in group E was significantly greater than in group C at 7 years ($p = 0.02$).

Six patients in group C had a total of 11 new vertebral fractures during the followup period (Table 3), whereas only 2 patients in group E had a total of 3 new fractures. At year 7, cyclic etidronate therapy had reduced the risk of new vertebral fracture by 67% [odds ratio (OR) 3.00; 95% confidence inter-

Table 1. Baseline characteristics at the beginning of the followup study (values are means ± SD).

Characteristics	Group E, n = 46	Group C, n = 45	p
Men, n(%)	6 (13)	8 (18)	NS
Premenopausal women, n (%)	25 (54)	24 (53)	NS
Postmenopausal women, n (%)	15 (33)	13 (29)	NS
Mean age, yrs			
Men	53 ± 17	43 ± 16	NS
Women	43 ± 14	43 ± 13	NS
Total corticosteroid dose, mg			
Men	2,577 ± 1245	1,705 ± 497	NS
Premenopausal women	2,497 ± 1816	1,852 + 506	NS
Postmenopausal women	2,143 ± 918	1,880 ± 931	NS
Lumbar spine BMD, g/cm^2			
Men	0.95 ± 0.17	0.90 ± 0.18	NS
Premenopausal women	0.86 ± 0.18	0.93 ± 0.14	NS
Postmenopausal women	0.78 ± 0.15	0.79 ± 0.14	NS

Group E: etidronate; Group C: control. BMD: bone mineral density; NS: not significant; SD: standard deviation.

Table 2. Average daily dose of PSL and ratio of patients still receiving corticosteroids (years after start of the prospective study).

Year		1	2	3	4	5	6	7
Average daily dose of PSL (mg/day, mean ± SD)	Group E	10.7 ± 3.8	9.4 ± 5.4	8.3 ± 3.8	7.7 ± 3.1	8.1 ± 4.0	9.3 ± 5.1	8.5 ± 4.4
	Group C	9.7 ± 3.7	8.1 ± 2.8	8.0 ± 3.3	7.8 ± 4.1	7.7 ± 4.1	8.0 ± 4.6	7.7 ± 3.4
	p value	NS	NS	NS	NS	NS	NS	NS
Ratio of patients receiving PSL (%)	Group E	100	100	100	98	95	92	92
	Group C	100	100	100	98	98	98	95
	p value	NS	NS	NS	NS	NS	NS	NS

Group E: etidronate; Group C: control. PSL: prednisolone; NS: not significant.

val 0.604–14.90; p = 0.18). The adjusted OR by menopausal status was calculated as 2.05.

There were no adverse events in group C, but 2 occurred in group E during the 3-year prospective study. However, there were no adverse events in either group during the followup period. Although serum calcium monitoring had not been performed systematically, where measured, no hypercalcemia was found in either group. None of the patients had gastrointestinal symptoms severe enough to discontinue the etidronate throughout the entire 7 years of followup in group E.

DISCUSSION

Our study demonstrated that longterm intermittent cyclical etidronate therapy increased the BMD of the lumbar spine in Japanese patients with CIOP. No significant reduction in risk of vertebral fractures at 7 years was achieved. However, this might be due to small sample size; future studies on larger numbers of patients will be required to draw a definitive conclusion on this point.

It has been reported before that etidronate increases BMD in CIOP[11-15], but there is only one report on longterm observation indicating efficacy of continuing etidronate therapy for more than 5 years in patients with asthma receiving oral and/or inhaled corticosteroids[9]. The longterm efficacy of intermittent etidronate therapy for CIOP in patients with CTD has to our knowledge never been evaluated. Ours is thus the first comprehensive study on the efficacy of longterm cyclical etidronate therapy of up to 7 years for CIOP in patients with CTD. Previous studies showed that alendronate or risedronate, which are both nitrogen-containing bisphosphonates, can maintain continuing increases in BMD, as well as effect a reduction of the fracture rate[4,16-19]. Our study is consistent with these findings with respect to maintaining BMD.

In our study, both groups C and E showed higher increases in lumbar spine BMD at years 6 and 7. In group C, it might be due to the fact that 9 patients added bisphosphonates besides activated vitamin D_3 during the followup study. Indeed, the mean percentage changes in patients who added bisphosphonates were higher compared with those patients who did not at year 7, although the differences were not statistically significant. In contrast, only 1 patient changed from etidronate to alendronate in group E. However, this patient and 2 patients who discontinued the corticosteroids during the followup study showed considerable increases in lumbar

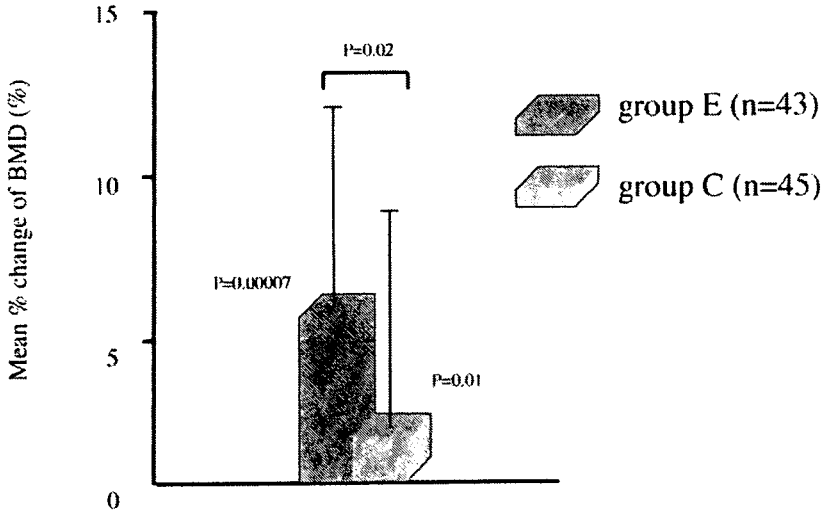

Figure 1. The mean (± SD) percentage change in bone mineral density (BMD) of the lumbar spine between baseline (0 yr) and 7 years of groups E and C. In all patients, the mean (± SD) percentage change in BMD of the lumbar spine increased 5.9% ± 8.8% (p = 0.00007) from baseline at 7 years in group E and 2.2% ± 5.8% (p = 0.013) in group C. The improvement of BMD in group E was significantly higher than in group C at 7 years (p = 0.02).

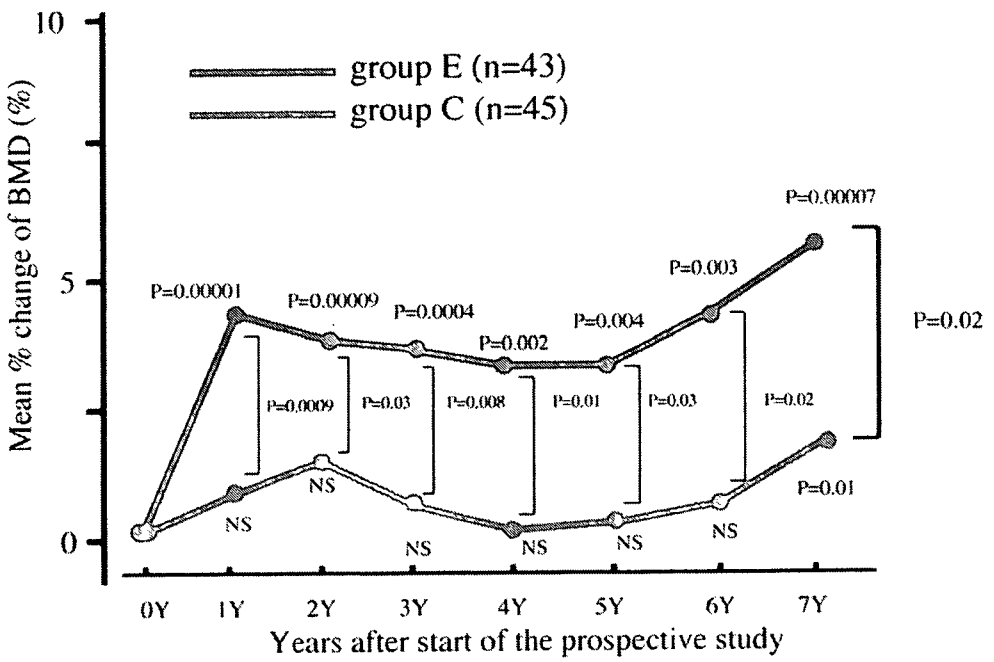

Figure 2. The mean percentage change in lumbar spine BMD in group E improved from baseline by approximately 5% over the 7-year study.

spine BMD at years 6 or 7. These increases in lumbar spine BMD might have contributed to the increase in the mean percentage change of lumbar spine BMD to some degree. As far as we know, there were no patients who took other medications. Persistent prescriptions for etidronate and activated vitamin D_3 have been confirmed in the medical records. Therefore, we think that other medications or compliance with treatment would not affect the BMD data in either group.

The ability of activated vitamin D_3 to prevent the loss of BMD caused by corticosteroids has been reported[20]. In our study, the control group (receiving activated vitamin D_3) slightly increased their lumbar spine BMD and maintained this for 7 years. However, as there was no placebo control in our study, we could not compare the rate of vertebral fracture in the group receiving activated vitamin D_3 to that of the placebo group.

Table 3. Incidence of vertebral fractures at 7 years in group E (etidronate) and C (control).

	Group E	Group C
Male	0/4	1/6
Female		
Premenopausal	1/24	1/23
Postmenopausal	1/10	4/13
Total	2/38	6/42
Total vertebral fracture	3	11

A randomized, double-blinded, multicenter study showed almost the same efficacy of either 200 mg or 400 mg of cyclical intermittent etidronate therapy in Japanese patients with involutional osteoporosis[21]. The current approved dose of etidronate for osteoporosis in Japan is 200 mg daily. When the dose of 200 mg of etidronate was ineffective or the patients had severe osteoporosis, 400 mg was used. Our results here indicated that 200 mg of etidronate was sufficiently effective to prevent or treat Japanese patients with CIOP. This might be due to racial differences or the difference in the dose per unit body weight between Japanese and Caucasian patients. Because our study did not include large numbers of patients, further observations will be needed to confirm this hypothesis.

Regarding adverse events, it was notable that severe side effects were not seen throughout the 7 years of the study. No gastrointestinal disorders, bone necrosis, or disturbance of bone formation was recorded. Only 2 patients had an adverse event of any kind in group E during the 3-year prospective study. No other patients dropped out of the study because of drug intolerance or discomfort. Treatment compliance was good and we can conclude that intermittent cyclical etidronate therapy is very well tolerated. Moreover, in view of its cost-effectiveness[22], cyclical intermittent etidronate therapy should be considered as a routine treatment option for CIOP, especially in patients with previous or current gastrointestinal disorders.

Intermittent cyclical etidronate therapy significantly increased BMD of the lumbar spine and maintained it over 7 years in patients with CIOP. There was a tendency towards a reduction in the incidence of vertebral fractures at 7 years in etidronate-treated patients compared to activated vitamin D_3-treated patients, but this did not achieve statistical significance. Longterm intermittent cyclical etidronate therapy is a safe, well tolerated, and effective therapy for the prevention and treatment of CIOP in Japanese patients with CTD.

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Excessive exposure to anionic surfaces maintains autoantibody response to β_2-glycoprotein I in patients with antiphospholipid syndrome

Yukie Yamaguchi,[1,2] Noriyuki Seta,[1] Junichi Kaburaki,[3] Kazuko Kobayashi,[4] Eiji Matsuura,[4] and Masataka Kuwana[1]

[1]Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo; [2]Department of Environmental Immuno-dermatology, Yokohama City University Graduate School of Medicine, Yokohama; [3]Department of Internal Medicine, Tokyo Electric Power Company Hospital, Tokyo; and [4]Department of Cell Chemistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

Antiphospholipid syndrome (APS) is an autoimmune prothrombotic disorder associated with autoantibodies to phospholipid (PL)–binding proteins, such as β_2-glycoprotein I (β_2GPI). We have recently reported that binding of β_2GPI to anionic PL facilitates processing and presentation of the cryptic β_2GPI epitope that activates pathogenic autoreactive T cells. To clarify mechanisms that induce sustained presentation of the dominant antigenic β_2GPI determinant in patients with APS, T-cell proliferation induced by β_2GPI-treated phosphatidylserine liposome (β_2GPI/PS) was evaluated in bulk peripheral blood mononuclear cell cultures. T cells from patients with APS responded to β_2GPI/PS in the presence of immunoglobulin G (IgG) anti-β_2GPI antibodies derived from APS plasma, and this response was completely inhibited either by the depletion of monocytes or by the addition of anti-FcγRI antibody. These findings indicate that efficient presentation of the cryptic determinants can be achieved by monocytes undergoing FcγRI-mediated uptake of β_2GPI-bound anionic surfaces in the presence of IgG anti-β_2GPI antibodies. Finally, β_2GPI-bound oxidized LDL or activated platelets also induced the specific T-cell response. Continuous exposure to these anionic surfaces may play a critical role in maintaining the pathogenic anti-β_2GPI antibody response in patients with APS. (Blood. 2007;110:4312-4318)

Introduction

Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by arterial and venous thrombosis as well as recurrent intrauterine fetal loss in the presence of antiphospholipid antibodies.[1] β_2-glycoprotein I (β_2GPI) is the most common antigenic target recognized by the antiphospholipid antibodies, and anti-β_2GPI antibodies are shown to be strongly associated with thrombosis and other clinical manifestations of APS.[2-4] β_2GPI is a plasma protein that binds various anionic substances, including phospholipids (PLs), lipoproteins, and activated platelets and endothelial cells.[5-7] Several lines of evidence accumulated from animal models suggest that anti-β_2GPI antibodies are directly involved in the pathogenic processes of APS.[8,9]

We have recently identified $CD4^+$ T cells responsive to β_2GPI in patients with APS.[10-12] β_2GPI-reactive T cells can promote production of pathogenic immunoglobulin G (IgG) anti-β_2GPI antibodies from autologous B cells in vitro. These T cells respond to bacterially expressed recombinant β_2GPI fragments and chemically reduced β_2GPI, but fail to respond to native β_2GPI,[10] indicating that the epitopes recognized by β_2GPI-reactive T cells are cryptic determinants that are not generated through processing of native β_2GPI under normal circumstances. One of the major cryptic determinants recognized by β_2GPI-reactive T cells is the region spanning amino acids (AAs) 276-290, which contains the major PL-binding site at AA 281-288,[13,14] in the context of HLA-DRB4*0103 (DR53).[11] In our recent study employing β_2GPI-reactive $CD4^+$ T-cell clones generated from patients with APS, dendritic cells or macrophages pulsed with β_2GPI-bound phosphatidylserine (PS) liposome induced a response of T-cell clones specific for a peptide encoding AA 276–290 (p276-290) in HLA-DR–restricted and antigen-processing–dependent manners. In contrast, those pulsed with β_2GPI or PS liposome alone failed to induce a response.[15] Together these findings indicate that specialized antigen-presenting cells (APCs) capturing β_2GPI-coated anionic PLs efficiently present a disease-relevant cryptic T-cell determinant of β_2GPI as a result of antigen processing.

In patients with APS, anti-β_2GPI antibody levels are usually stable for many years. However, it remains unclear what mechanisms are responsible for the sustained presentation of the dominant cryptic β_2GPI determinant that activates β_2GPI-reactive T cells to subsequently produce pathogenic anti-β_2GPI antibodies. To elucidate these mechanisms, we examined the cellular and molecular factors required for the sustained activation of β_2GPI-reactive T cells in patients with APS.

Patients, materials, and methods

Patients and controls

This study examined 5 patients, and all fulfilled the revised Sapporo criteria for APS proposed by the International Workshop.[16] These patients were selected based on the presence of DRB4*0103 (DR53), which is known to present a p276-290 peptide to T cells,[11] and positive IgG anti-β_2GPI antibody. The HLA class II alleles, including DRB1 and DRB4, were determined by restriction fragment length polymorphisms combined with

Submitted July 9, 2007; accepted August 23, 2007. Prepublished online as *Blood* First Edition paper, August 28, 2007; DOI 10.1182/blood-2007-07-100008.

An Inside *Blood* analysis of this article appears at the front of this issue.

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locus-specific polymerase chain reaction using peripheral blood granulocyte-derived genomic DNA as a template.[17] IgG anti-β_2GPI antibody levels were measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Yamasa, Choshi, Japan) using immobilized β_2GPI-cardiolipin complex as an antigen source. A commercial kit based on Russell viper venom test (Gradipore, Sydney, Australia) was used to determine the presence of lupus anticoagulant. At the time of blood examination, all the patients were taking low-dose corticosteroids (< 10 mg/day) and low-dose aspirin. Peripheral blood from healthy volunteers was also used as a control source of plasma. All samples were obtained after the patients and control subjects gave their written informed consent in accordance with the Declaration of Helsinki. The study protocol was approved by Keio University International Review Board.

Antigen preparations

Human β_2GPI was purified from normal pooled plasma,[18] and reduced β_2GPI was prepared by incubating β_2GPI with dithiothreitol as previously described.[10] We generated a panel of recombinant maltose-binding protein (MalBP) fusion proteins expressing full-length β_2GPI (GP-F), domains I and II (GP1), domains III and IV (GP2), and domains IV and V (GP3).[10] MalBP alone was prepared as a control antigen. Two 15-mer peptides, p276-290 and a peptide encoding AA 306-320 of human β_2GPI (p306-320), were synthesized using a solid-phase multiple synthesizer (Advanced ChemTech, Louisville, KY).[11]

Liposome containing bovine brain-derived PS (Sigma, St Louis, MO), with a composition of dioleoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) at a molar ratio of 3:7, was prepared and adjusted to a final concentration of 1 μmol/mL.[19,20] Low density lipoprotein (LDL) was isolated from freshly prepared normal human plasma by ultracentrifugation, and oxidized LDL (oxLDL) was prepared by incubating LDL with 5 μM $CuSO_4$ for 8 hours at 37°C.[20] LDL and oxLDL were adjusted to 100 μg/mL of apoB equivalent. Human platelets were separated from platelet-rich plasma using a modified gel filtration method[21] to minimize their activation during an isolation procedure. Resting platelets were then activated by incubation with bovine thrombin (1 U/mL; Mochida, Tokyo, Japan) for 15 minutes. All preparations were incubated with or without native β_2GPI (100 μg/mL) for 30 minutes at room temperature immediately prior to use in the cultures.

Cell preparations

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway) density-gradient centrifugation. In some experiments, PBMCs were depleted of $CD14^+$ monocytes or $CD19^+$ B cells by incubation with anti-CD14 or anti-CD19 monoclonal antibody (mAb)–coupled magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany), respectively, followed by magnetic cell sorting column separation according to the manufacturer's protocol.

Preparation and depletion of IgG from plasma

The IgG fraction was purified or depleted from plasma samples using HiTrap protein G (Amersham Biosciences, Uppsala, Sweden) as described previously.[22] Purity of IgG fractions was confirmed to be more than 95% by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by densitometry on Coomassie blue–stained gels. In some experiments, purified IgG was treated with pepsin to prepare $F(ab')_2$ using a Fab2 preparation kit (Pierce Biotechnology, Rockford, IL). We also prepared IgG fractions depleted of antibodies specific to β_2GPI. Briefly, purified IgG samples were treated 3 times with cardiolipin-coated 96-well immunoplates (Nunc F96Maxisorp, Roskilde, Denmark), which were preincubated with β_2GPI or phosphate-buffer saline for 30 minutes. The supernatants were then collected as anti-β_2GPI antibody–depleted or mock-treated IgG. Removal of anti-β_2GPI antibody was confirmed by complete loss of antibody reactivity on the anti-β_2GPI antibody ELISA.

Assays for antigen-specific T-cell response

Antigen-specific T-cell proliferation in the primary cultures was assayed as described previously[10] with some modifications. Briefly, PBMCs (10^5/well) were cultured with or without antigen in 96-well flat-bottomed culture plates for 7 days. RPMI 1640 supplemented with either 10% fetal bovine serum (FBS; JRH Bioscience, Lenexa, KS) or 8% platelet-poor plasma, which was derived from patients with APS and healthy donors, was used as medium. Prior to use, FBS and plasma samples were heat-inactivated and depleted of β_2GPI by passing the samples through a HiTrap Heparin column (Amersham Biosciences) twice, to eliminate the potential influence of intrinsic β_2GPI on the generation of the antigenic peptides. ^{3}H-thymidine (0.5 μCi [0.0185 MBq]/well) was added to the cultures during the final 16 hours. The cells were harvested, and ^{3}H-thymidine incorporation was measured in a Top-Count microplate scintillation counter (Packard, Meriden, CT). Native β_2GPI, reduced β_2GPI, GP-F, GP1, GP2, GP3, and MalBP were used as antigens at a concentration of 10 μg/mL. In addition, PS liposome (0.1 μmol/mL), LDL, oxLDL (10 μg/mL apoB equivalent), resting platelets, or activated platelets (10^6/well) were added to the cultures, with or without preincubation with β_2GPI. To exclude nonspecific unresponsiveness of T cells, all experiments included a culture with phytohemagglutinin at a final concentration of 1 μg/mL. In some experiments, purified IgG, $F(ab')_2$, or anti-β_2GPI antibody–depleted or mock-treated IgG was added at the initiation of the culture. Anti-FcγRI (clone 10.1; R&D Systems, Minneapolis, MN), anti-HLA-DR (clone L243; Leinco Technologies, Baldwin, MO), or isotype-matched control mAb was also added to the culture at a final concentration of 2.5 μg/mL. All experiments were carried out in duplicate or triplicate, and the values are the mean counts per minute (cpm) plus or minus the standard deviation of multiple determinations. In some instances, a T-cell response specific to β_2GPI-treated PS liposome (β_2GPI/PS) was expressed as the ratio of cpm in the culture with β_2GPI/PS to cpm in the culture with PS liposome alone.

Secondary stimulation of peripheral blood T cells was also performed as described.[10] PBMCs were primed with β_2GPI/PS in medium supplemented with 8% autologous plasma for 10 days. Viable cells were then cultured for an additional 3 days in the presence of 50 U/mL recombinant interleukin-2 (Biogen Idec, San Diego, CA) and irradiated (3000 rad) autologous monocyte-derived dendritic cells in medium supplemented with 10% FBS in the absence or presence of β_2GPI, reduced β_2GPI, GP-F, GP1, GP2, GP3, MalBP (10 μg/mL), p276-290, or p306-320 (5 μg/mL). Frequencies of β_2GPI-reactive T cells in peripheral blood T cells were estimated by limiting dilution analysis using GP-F as an antigen.[23] The recognition of p276-290 by peripheral blood T cells was determined based on the specific response to p276-290 by at least 2 T-cell clones established by repeated stimulation of peripheral blood T cells with GP-F.[11]

Results

Clinical and immunologic characteristics of patients with APS

As shown in Table 1, all patients with APS had thrombosis and/or loss of pregnancy, and were positive for lupus anticoagulant. IgG anti-β_2GPI antibody titer was high in all but one patient (APS1). Frequencies of β_2GPI-reactive T cells were variable among patients, and ranged from 2.9 to 12.4 per 10^4 peripheral blood T cells. In addition, T-cell recognition of p276-290 was detected in all 3 patients examined.

T-cell response induced by β_2GPI/PS in PBMC cultures

We first examined the responses of peripheral blood T cells to β_2GPI/PS using regular medium supplemented with FBS (Figure 1A). T cells from all 5 patients responded to GP-F, but failed to proliferate in the presence of β_2GPI/PS. Interestingly, a T-cell response to β_2GPI/PS, as well as to GP-F, was detected when a patient's autologous plasma was used instead of FBS to supplement the culture medium. This response was blocked by anti-HLA-DR

Table 1. Clinical and immunologic characteristics of patients with APS analyzed in this study

Patient no.	Age/sex	Thrombosis	Loss of pregnancy	IgG anti-β_2GPI antibodies (U/mL)†	HLA class II alleles: DRB1	Frequency of β_2GPI-reactive T cells in circulation/10^4 T cells	Recognition of p276–290 by peripheral blood T cells
APS1	51/F	None	+	16	*1502/*0405	4.5	NT
APS2	43/F	DVT, stroke	+	>120	*0405/*1202	2.9	NT
APS3	46/F	DVT, PE, retinal artery thrombosis	+	>120	*1502/*0901	6.8	+
APS4	47/F	Stroke	+	>120	*1501/*0403	8.1	+
APS9	46/F	DVT, PE, stroke, amaurosis fugax	NA	>120	*0901	12.4	+

All patients were lupus anticoagulant positive; all DRB4 alleles were *0103.
DVT indicates deep venous thrombosis of lower extremity; PE, pulmonary embolism; NA, not applicable; and NT, not tested.
†Normal range less than 3.5 U/mL.

mAb, but not by control mAb (data not shown). However, a β_2GPI/PS-induced response was not detected in the culture with allogenic plasma from a healthy individual. This finding was reproducible in a total of 7 PBMC samples obtained from 5 patients with APS.

Next, PBMCs from a patient with APS were cultured with β_2GPI/PS or PS liposome alone in medium supplemented with 2 different lots of FBS, plasma samples from 4 patients with APS, or samples from 3 healthy donors (Figure 1B). The β_2GPI/PS-specific response was exclusively detected in cultures with autologous and allogenic plasmas derived from patients with APS, although the degree of response was variable among APS plasmas. Analogous findings were obtained with PBMCs from 3 additional patients with APS. In all cases, the lowest response was detected in the culture supplemented with APS1 plasma, which contained low-titer anti-β_2GPI antibodies.

We next sought to confirm whether T-cell responses induced by β_2GPI/PS in cultures with APS plasma were specific to β_2GPI. Peripheral blood T cells primed with β_2GPI/PS in medium supplemented with autologous plasma were further examined for their reactivity to various β_2GPI preparations in the secondary culture with FBS (Figure 2). β_2GPI/PS-primed T cells from all 5 patients specifically responded to reduced β_2GPI, GP-F, and GP3, indicating a specific recognition of β_2GPI-derived peptides. More important, the cryptic p276-290 was efficiently presented by APCs in culture with β_2GPI/PS and APS plasma. T-cell recognition of GP1 was detected in APS2, APS4, and APS9 samples, whereas recognition of GP2 was detected in APS3 and APS9. Taken together, these findings together indicate that a soluble factor(s) contained in plasma from patients with APS, but not in FBS or plasma from healthy individuals, plays an essential role in activation of β_2GPI-specific T cells in bulk PBMC cultures with β_2GPI/PS.

IgG anti-β_2GPI autoantibody as an essential factor for T-cell recognition of β_2GPI/PS

Since the degree of the β_2GPI/PS-specific T-cell response appeared to correlate with IgG anti-β_2GPI antibody titers, we hypothesized that IgG anti-β_2GPI antibodies in APS plasma are required for peripheral blood T cells to respond to β_2GPI/PS. To test this hypothesis, we first prepared IgG-depleted APS plasma samples to evaluate the β_2GPI/PS-induced T-cell response (Figure 3A). Depletion of IgG from APS plasma resulted in complete loss of the β_2GPI/PS-induced T-cell response, but addition of autologous IgG back to the IgG-depleted APS plasma restored the response in a dose-dependent fashion. In contrast, addition of IgG prepared from healthy plasma had no effect (data not shown). Interestingly, β_2GPI/PS-induced T-cell response was also detected in medium supplemented with healthy plasma in the presence of IgG derived from APS plasma. This response was abolished when $F(ab')_2$ was used instead of intact IgG, indicating an important role of the Fc portion of IgG.

We further examined the effects of depletion of β_2GPI-specific antibody on the β_2GPI/PS-induced T-cell response in PBMC cultures with APS IgG (Figure 3B). β_2GPI/PS-induced T-cell

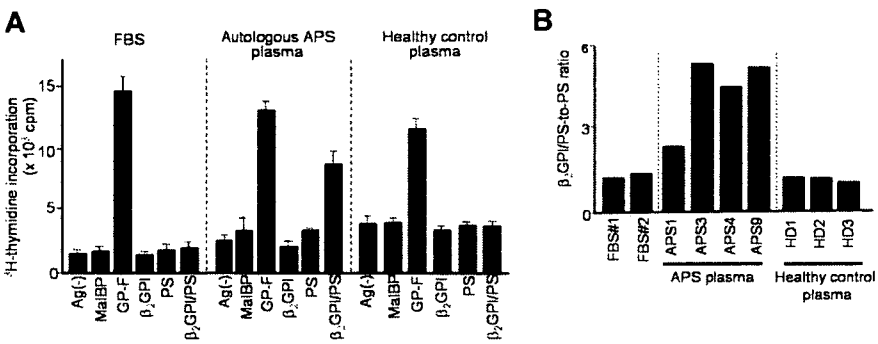

Figure 1. T-cell response to β_2GPI/PS in bulk PBMC cultures supplemented with FBS, autologous APS plasma, or healthy control plasma. (A) PBMCs from APS4 were cultured in triplicate with or without antigens, including MalBP, GP-F, β_2GPI, PS, and β_2GPI/PS, in medium supplemented with FBS, autologous APS plasma, or healthy control plasma. The antigen-induced T-cell proliferative response was assessed by ^{3}H-thymidine incorporation. Results are shown as mean (column) and standard deviation (error bar) of triplicate measurements. Analogous findings were obtained in 7 independent experiments in PBMCs from all 5 patients with APS. (B) β_2GPI/PS-specific T-cell response in PBMC cultures of APS4 in medium supplemented with 2 different lots of FBS (no. 1 and no. 2), plasma samples from 4 APS patients (APS1, 3, 4, and 9), or plasma samples from 3 healthy donors (HD1, 2, and 3). β_2GPI/PS-specific T-cell response was expressed as a β_2GPI/PS-to-PS ratio, which was the mean cpm incorporated in the triplicate culture with β_2GPI/PS divided by the mean cpm incorporated in the triplicate culture with PS alone (standard deviations for the individual results were within 20% of the mean in all cases). Similar results were obtained from 3 additional patients with APS (APS1, APS3, and APS9).

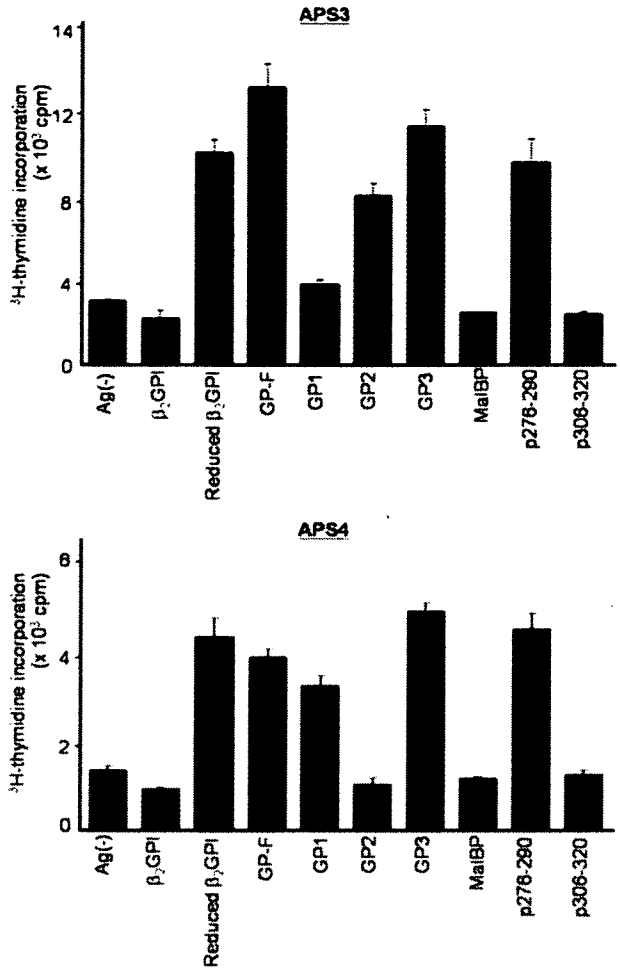

Figure 2. Proliferative responses of β_2GPI/PS-primed T cells to various β_2GPI preparations in secondary cultures. PBMCs from APS3 (top) and APS4 (bottom) were stimulated with β_2GPI/PS for 10 days in medium supplemented with autologous plasma. The viable T cells were then cultured in duplicate with β_2GPI, reduced β_2GPI, GP-F, GP1, GP2, GP3, MalBP, p276-290, or p306-320 in medium containing FBS. After 3 days, ^{3}H-thymidine incorporation was measured. Results are shown as mean (column) and standard deviation (error bar) of duplicate measurements.

response was detected in the presence of mock-treated APS IgG, but completely abolished by depletion of β_2GPI-reactive IgG. These findings indicate that IgG anti-β_2GPI antibodies are required for the T cells of patients with APS to respond to β_2GPI/PS in bulk PBMC cultures.

Roles of β_2GPI/PS-containing immune complex in β_2GPI/PS-induced T-cell response

Since anti-β_2GPI antibodies in sera from patients with APS recognize β_2GPI/PS,[20] it is likely that β_2GPI/PS is readily opsonized by IgG anti-β_2GPI antibodies in culture with APS plasma. To evaluate which APCs contained in PBMCs capture this immune complex to induce a specific T-cell response to β_2GPI peptides, we analyzed PBMCs depleted of CD14$^+$ monocytes, CD19$^+$ B cells, or mock-treated in cultures with β_2GPI/PS and autologous plasma (Figure 4A). The β_2GPI/PS-induced T-cell response was completely inhibited by depletion of monocytes, but was partially suppressed by depletion of B cells, suggesting a primary role of monocytes in our system.

We further evaluated the potential involvement of Fcγ receptors in recognition of the immune complex by monocytes, as the anti-β_2GPI F(ab')$_2$ was incapable of inducing the T-cell response to β_2GPI/PS. The β_2GPI/PS-induced T-cell response was completely blocked by anti-FcγRI mAb, but not by control mAb (Figure 4B). Together these findings indicate that efficient β_2GPI/PS-induced T-cell response is achieved by monocytes undergoing FcγRI-mediated uptake of β_2GPI/PS opsonized by IgG anti-β_2GPI autoantibodies.

T-cell response to β_2GPI-treated oxLDL and platelet microparticles

PS liposomes were chemically synthesized, and may not be relevant to patients with APS in vivo. To examine whether anionic substances present in the circulation, such as oxLDL or platelet microparticles, can substitute for PS liposomes in inducing the β_2GPI-specific T-cell response, PBMCs from a representative patient with APS were cultured with various anionic and control substances pretreated with or without β_2GPI in medium supplemented with autologous plasma (Figure 5). OxLDL or activated platelets pretreated with β_2GPI induced a T-cell proliferative

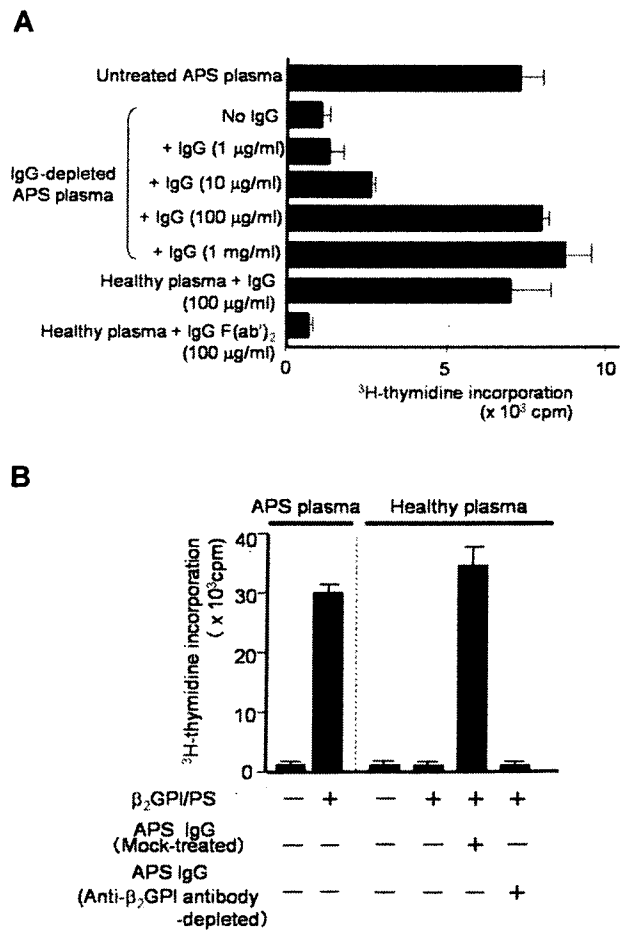

Figure 3. β_2GPI/PS-induced T-cell response in PBMC cultures with or without IgG derived from APS plasma. (A) PBMCs obtained from APS3 were cultured in triplicate with β_2GPI/PS in medium supplemented with untreated or IgG-depleted autologous APS plasma, or healthy plasma. Purified IgG (1 μg/mL-1 mg/mL) or IgG F(ab')$_2$ (100 μg/mL) from APS3 was added to the cultures. After 7 days, the T-cell proliferative response induced by β_2GPI/PS was measured by ^{3}H-thymidine incorporation. Results are shown as mean (column) and standard deviation (error bar). Concordant results were obtained with a sample from APS4. (B) PBMCs derived from APS3 were cultured in triplicate with or without β_2GPI/PS in medium supplemented with autologous APS plasma or healthy plasma. An anti-β_2GPI antibody–depleted or mock-treated autologous IgG fraction was added to the initiation of cultures. After 7 days, the T-cell proliferative response was measured by ^{3}H-thymidine incorporation. Results are shown as mean (column) and standard deviation (error bar). Concordant results were obtained with a sample from APS4.

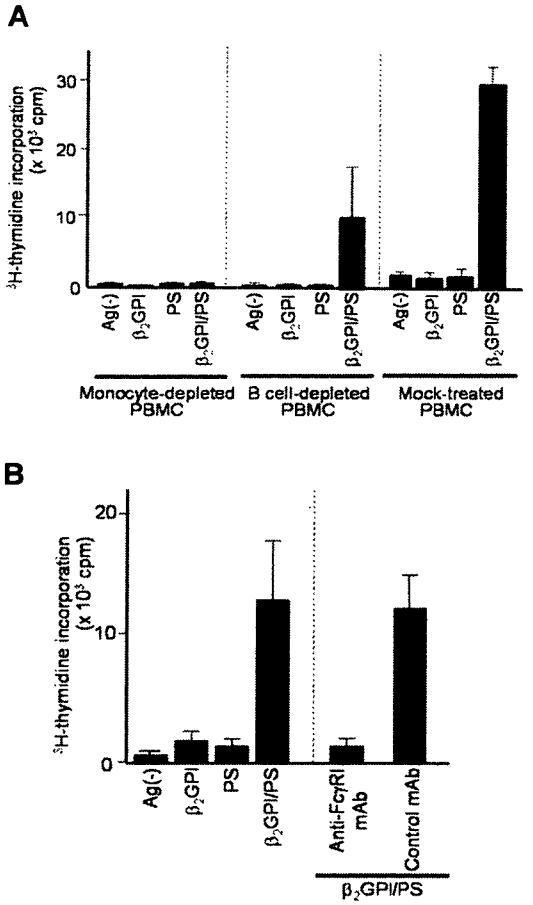

Figure 4. Effects of APC depletion or anti-FcγRI mAb on β_2GPI/PS-induced T-cell response. (A) CD14$^+$ monocyte-depleted, CD19$^+$ B-cell–depleted, and mock-treated PBMCs derived from APS3 were cultured for 7 days with or without β_2GPI, PS, or β_2GPI/PS in medium supplemented with autologous APS plasma, and the T-cell proliferative response was measured by ^{3}H-thymidine incorporation. Results are shown as mean (column) and standard deviation (error bar) of duplicate measurements. Analogous results were obtained in a total of 4 independent experiments using samples from 3 patients with APS (APS1, APS3, and APS4). (B) PBMCs from APS2 were cultured for 7 days with or without β_2GPI, PS, or β_2GPI/PS in medium supplemented with autologous APS plasma. Anti-FcγRI or isotype-matched control mAb was added to the initiation of cultures. The T-cell proliferative response was evaluated by ^{3}H-thymidine incorporation. Results are shown as mean (column) and standard deviation (error bar) of duplicate measurements. Concordant results were obtained with samples from 3 patients with APS (APS2, APS3, and APS9).

response, as observed in cultures with β_2GPI/PS. These responses were specifically inhibited by anti–HLA-DR mAb (data not shown). Thus, oxLDL and activated platelets can be in vivo sources of anionic surfaces that bind β_2GPI and promote the efficient presentation of β_2GPI cryptic peptides by APCs.

Discussion

This study evaluated the potential cellular and molecular mechanisms that induce sustained presentation of the dominant cryptic β_2GPI determinant that activates β_2GPI-reactive T cells to subsequently produce pathogenic anti-β_2GPI antibodies in patients with APS. Here we demonstrate that efficient presentation of cryptic determinants recognized by β_2GPI-reactive T cells is achieved by monocytes undergoing FcγRI-mediated uptake of β_2GPI/PS opsonized by IgG anti-β_2GPI antibodies. High avidity IgG anti-β_2GPI antibodies, which were reported to possess high pathogenicity,[24] would also have enhanced capacity to promote this process. We

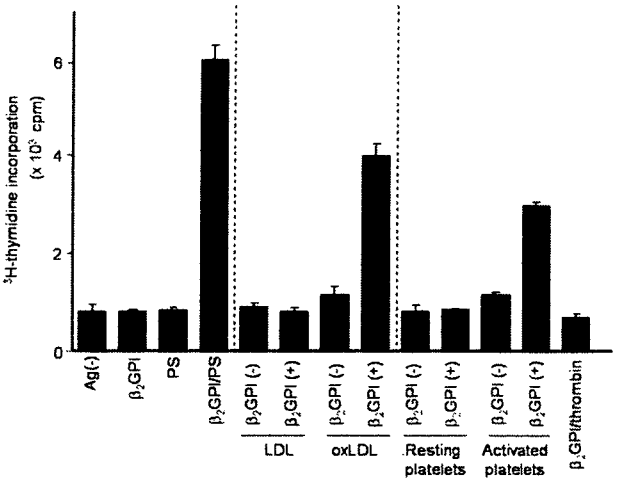

Figure 5. T-cell responses to β_2GPI-treated anionic substances present in circulation. PBMCs from APS4 were cultured with or without various antigen preparations in medium supplemented with autologous APS plasma. Antigens used included β_2GPI alone, as well as PS, LDL, oxLDL, resting platelets, and activated platelets, which were treated either with or without β_2GPI. Thrombin, which was used to activate platelets, in combination with β_2GPI served as a control. T-cell proliferative response was measured by ^{3}H-thymidine incorporation. Results are shown as mean (column) and standard deviation (error bar) of duplicate measurements. Analogous results were obtained in samples from all 5 patients with APS.

propose a model by which a pathogenic loop maintains sustained anti-β_2GPI autoantibody production in patients with APS (Figure 6). This model consists of 3 major players: β_2GPI-reactive CD4$^+$ T cells, anti-β_2GPI antibody-producing B cells, and macrophages. Upon recognition of β_2GPI cryptic peptides, such as p276-290, presented by macrophages in the context of HLA-DR, β_2GPI-reactive CD4$^+$ T cells are activated and exert helper activity that induces IgG anti-β_2GPI antibody production from B cells. This process can be achieved by T-B cell collaboration through CD40-CD154 engagement and T cell–derived IL-6.[11] IgG anti-β_2GPI antibodies subsequently recognize β_2GPI-bound anionic surfaces in circulation, resulting in enhanced phagocytosis of this immune

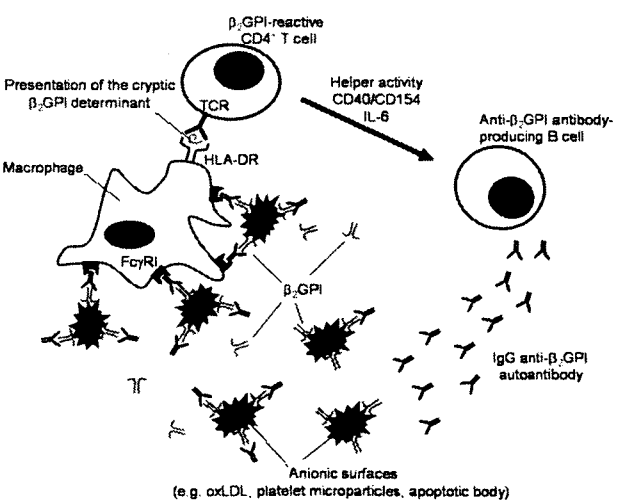

Figure 6. A schematic model representing a continuous autoimmune loop carried out by macrophage, β_2GPI-reactive CD4$^+$ T cell, and anti-β_2GPI antibody–producing B cell. The macrophage efficiently presents the cryptic β_2GPI determinant in the context of HLA-DR. The β_2GPI-reactive CD4$^+$ T cell is activated by recognition of the cryptic β_2GPI peptide and exerts helper activity that induces production of IgG anti-β_2GPI autoantibodies from the specific B cell. The immune complex consisting of anionic surfaces, β_2GPI, and IgG anti-β_2GPI antibodies were captured by macrophages via FcγRI.

complex by macrophages through FcγRI. In this regard, it has been shown that anti-β_2GPI antibodies in APS sera are predominantly of IgG2 subclass,[25,26] which has low affinity to FcγRI. However, anti-β_2GPI antibodies of IgG1 or IgG3 subclass were also detected in many patients with APS. These low levels of anti-β_2GPI antibodies with high binding affinity to FcγRI may be sufficient to drive the pathogenic loop. We have previously shown that β_2GPI binding to anionic substances promotes the generation of β_2GPI cryptic peptides by protecting the major PL-binding site from protease attack during antigen-processing by dendritic cells or macrophages.[15] Since it has been shown that antibody binding to the antigen boosts the generation of some minor epitopes,[27] binding of IgG anti-β_2GPI antibodies to the β_2GPI-anionic substance complex may further amplify generation of previously cryptic β_2GPI peptides. Moreover, this immune complex is likely to stimulate monocytes via FcγRI to secrete tissue factor, which is shown to play an important role in thrombus formation in patients with APS.[28] Partial suppression of the β_2GPI/PS-induced T-cell response by depletion of B cells in our system suggests that presentation of cryptic β_2GPI peptides could be mediated through B cells that capture β_2GPI/PS via specific B-cell receptors. This process, however, might have less of an impact on the T-cell response, due likely to low abundance of specific B cells recognizing β_2GPI/PS. The mechanism that triggers anti-β_2GPI antibody response in patients with APS remains unclear, but once this autoimmune loop is established, pathogenic anti-β_2GPI antibodies are continuously produced.

The presence of anionic substances with the capacity to bind β_2GPI is essential to drive the pathogenic loop inducing continuous anti-β_2GPI antibody production in patients with APS. Potential anionic substances in the circulation include apoptotic bodies, microparticles derived from activated platelets and endothelial cells, and oxLDL. Since β_2GPI is abundantly present in the circulation (~200 μg/mL), excessive exposure to anionic substances would result in the immediate formation of a complex with β_2GPI. In the present study, we have clearly demonstrated that microparticles derived from activated platelets and oxLDL can function as a substitute for the PS liposome that binds to β_2GPI and facilitates presentation of the cryptic epitopes of β_2GPI as a consequence of antigen processing. In addition, some of our group (E.M. and K.K.) reported that stable and nondissociable β_2GPI-oxLDL complexes were frequently detected in sera from patients with APS and/or systemic lupus erythematosus, but not in healthy individuals.[29] In addition, β_2GPI is known to have antiatherosclerosis activity by preventing oxLDL uptake by macrophages via scavenger receptor, but binding of IgG anti-β_2GPI antibodies to β_2GPI-oxLDL complexes mediates atherosclerosis by promoting phagocytosis of macrophages via Fcγ receptor.[29-31] Furthermore, elevated levels of procoagulant microparticles were detected in patients with APS in association with anti-β_2GPI antibodies and lupus anticoagulant.[32-34] The presence of a large quantity of anionic substances in circulation in patients with APS supports our proposed model.

Based on our model, therapeutic strategies that inhibit pathogenic anti-β_2GPI antibody production should target interrupting the continuous autoimmune loop carried out by macrophages and β_2GPI-reactive CD4$^+$ T cells and B cells. These immune cells are already targets of therapies under consideration, such as the anti-CD20 chimeric antibody rituximab.[35] Another potential therapeutic approach includes the removal of immune complexes consisting of β_2GPI, anionic substance, and anti-β_2GPI antibodies. Accordingly, plasma exchange and double filtration plasmapheresis, which theoretically remove such immune complexes, are shown to be effective for patients with intractable APS, including catastrophic APS.[36,37] Alternatively, small molecules that inhibit Fc receptor downstream signaling would have beneficial effects in patients with APS by suppressing the generation of β_2GPI cryptic peptides.[38]

In summary, excessive exposure to anionic surfaces may play a key role in maintaining the pathogenic anti-β_2GPI antibody response in patients with APS. Further studies should focus on mechanisms that prime the autoimmune loop and development of novel therapeutic strategies targeting the pathogenic process.

Acknowledgments

We thank Yuka Okazaki and Takahide Arai for expert technical assistance.

This work was supported by a grant from the Japanese Ministry of Health, Welfare, and Labor, and a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports and Culture.

Authorship

Contribution: Y.Y., N.S., J.K., K.K., and E.M. performed experiments; Y.Y. and M.K. analyzed results and made the figures; Y.Y. and M.K. designed the research and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Masataka Kuwana, Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan; e-mail: kuwanam@sc.itc.keio.ac.jp.

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