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The Future of Stem Cell Transplantation in Autoimmune Disease

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Abstract We have previously found that conventional allogeneic bone marrow transplantation (allo BMT) can be used to treat various spontaneously developed autoimmune diseases in mice. In addition, we have found that autoimmune diseases can be transferred into the normal mice by conventional BMT from autoimmune-prone mice. Based on these findings, we have proposed that autoimmune diseases are "stem cell disorders." To apply allo BMT to humans, we extensively carried out BMT to clarify which cells are essential for successful BMT, and finally found that three types of cells are essential for successful allogeneic BMT: (1) hemopoietic stem cells (HSCs), (2) natural suppressor cells, and (3) stromal cells (including mesenchymal stem cells, MSCs). We have very recently found that MSCs play a crucial role in preventing graft failure, since there is a major histocompatibility complex restriction between HSCs and MSCs. To recruit donor-derived MSCs, we have found that the injection of whole bone marrow cells into the bone marrow cavity (intra-bone marrow-BMT, IBM-BMT) is the best strategy for the treatment of various otherwise intractable diseases, including autoimmune diseases. In this review article, we provide evidence that IBM-BMT heralds a revolution in the field of transplantation and regeneration medicine.

Keywords Bone marrow transplantation (BMT) · Autoimmune disease · Intra-bone marrow (IBM)-BMT · Hemopoietic stem cell (HSC) · Mesenchymal stem cell (MSC)

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Introduction

In 1985, we found that allogeneic (but not syngeneic or autologous) bone marrow transplantation (BMT) could be used to treat autoimmune diseases in autoimmune-prone mice [1, 2]. Since then, we have confirmed our findings using a variety of autoimmune-prone mice [3–5].

Conversely, we have succeeded in inducing autoimmune diseases in normal mice by transplanting T-cell-depleted bone marrow cells (BMCs) or partially purified hemopoietic stem cells (HSCs) from autoimmune-prone mice to normal mice [6, 7]. Based on these findings, we proposed that autoimmune diseases were "stem cell disorders (SCDs)" [6–8].

Our findings have also been confirmed in humans: patients with autoimmune diseases were cured after allogeneic BMT, while autoimmune diseases were found to be transferred to recipients of BMT from donors who were suffering from autoimmune diseases [9].

In this article, we show that various otherwise intractable diseases (including autoimmune diseases) can be cured by our novel BMT method.

Strategies for recruitment of donor stromal cells

Using radiosensitive and chimeric-resistant MRL/lpr mice, we have found that the recruitment of donor stromal cells is essential for successful allogeneic BMT. We found that three methods are effective in replacing recipient stromal cells with donor-derived stromal cells: (1) conventional intravenous BMT (IV-BMT) plus bone grafts [10, 11]; (2) BMT from the portal vein (PV-BMT) [12], since tolerance can be easily induced when the antigen is portal venously (PV) injected into the liver; and finally (3) intra-bone

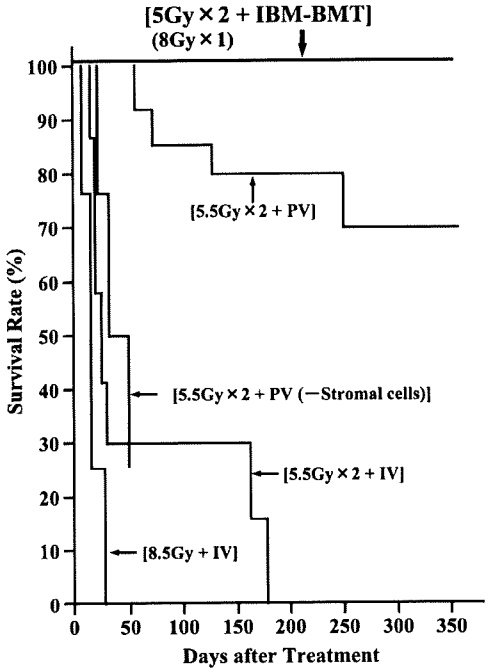

Fig. 1 Treatment of autoimmune diseases in MRL/lpr mice by IBM-BMT (5 Gy×2). IBM-BMT can be used to treat autoimmune diseases in MRL/lpr mice even when the radiation dose is reduced to 5 Gy×2

marrow (IBM)-BMT [13]. IBM-BMT was ultimately found to be best, since it allows us to use a mild conditioning regimen (5 Gy×2), as shown in Fig. 1. We therefore used IBM-BMT instead of the conventional IV-BMT in subsequent experiments.

IBM-BMT for organ transplantation

Since we have previously found that the combination of organ allografts and conventional IV-BMT from the same donors prevents the rejection of organ allografts [14], we attempted to apply IBM-BMT to organ allografts. IBM-BMT was the most effective strategy, since the radiation dose could be reduced to 4.0 Gy×2 in skin allografts [15]. In addition, we found that IBM-BMT is applicable to allografts of other organs and tissues in rats, such as pancreas islets [16], legs [17], lungs [18], and heart [19].

IBM-BMT for regeneration therapy

As it was apparent that donor stromal cells could be effectively recruited by "IBM-BMT", we next attempted to treat osteoporosis in SAMP6 mice; the SAMP6 mouse (a substrain of senescence-accelerated mice) spontaneously develops osteoporosis early in life and is therefore a useful model for examining the mechanisms underlying osteoporosis. After IBM-BMT, the hematolymphoid system was completely reconstituted with donor-type cells. Thus-treated SAMP6 mice (8 months after IBM-BMT) showed marked increases in trabecular bone even at 20 months of age (Fig. 2) and the bone mineral density remained similar to that of normal B6 mice. Bone marrow stromal cells in "IBM-BMT"-treated SAMP6 mice were replaced by donor stromal cells [20, 21]. Thus, we succeeded in curing osteoporosis in SAMP6 mice by IBM-BMT, which can recruit both donor- derived HSCs and MSCs.

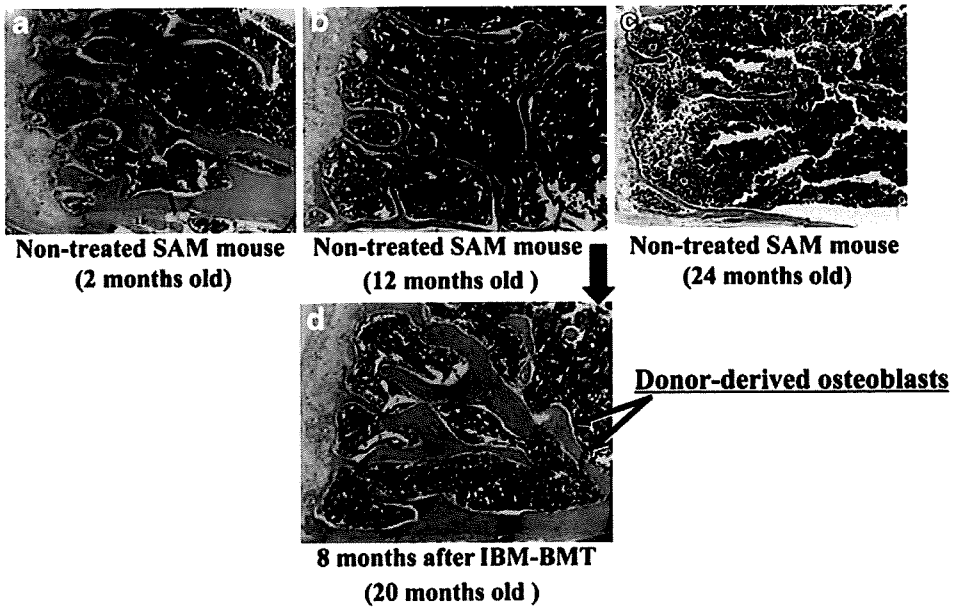

Fig. 2 Treatment of osteoporosis in SAMP6 mice by IBM-BMT from normal B6 mice

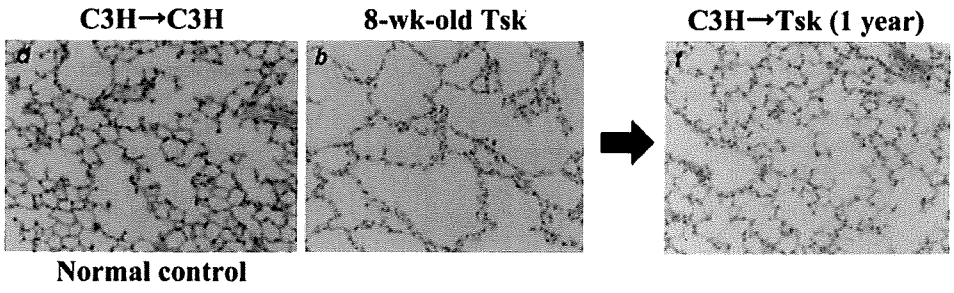

Fig. 3 Amelioration of emphysema in [C3H→Tsk] mice by IBM-BMT

Since IBM-BMT appeared to be a powerful strategy in regeneration therapy, we next used tight-skin (Tsk) mice (an animal model for emphysema) to examine whether emphysema could be cured by IBM-BMT.

IBM-BMT was carried out from C3H mice into Tsk mice (8–10 weeks old) that had already shown emphysema. Eight months after the transplantation, the lungs of all the Tsk mice treated with IBM-BMT [C3H→Tsk] showed structures similar to those of normal mice, whereas the [Tsk→Tsk] mice showed emphysema, as seen in age-matched Tsk mice (Fig. 3). Next, we attempted to transfer emphysema from Tsk mice to C3H mice by IBM-BMT. Six months after IBM-BMT, the [Tsk→C3H] mice showed emphysema [22]. These results strongly suggested that emphysema in Tsk mice originates from defects in the stem cells (probably MSCs and/or HSCs) in the bone marrow [22].

IBM-BMT + donor lymphocyte infusion for treatment of malignant tumors

It is well known that the graft-versus-leukemia reaction can cure patients of a variety of hematological malignancies [23, 24]. Recently, it has been reported that graft-versus-tumor (GvT) effects can induce partial (complete in some) remission of metastatic solid tumors such as breast cancer [14, 25, 26] and renal cell carcinoma [27–33]. Based on these findings, donor lymphocyte infusion (DLI) has recently been used for the treatment of malignant solid tumors even in humans. However, it is very difficult to completely eradicate the tumors, since extensive DLI induces graft-versus-host disease (GvHD). We therefore attempted to establish a new method for the treatment of malignant tumors, this method consisting of intra-bone marrow-IBM-BMT plus DLI, since we have recently found that IBM-BMT can allow a reduction in radiation doses as a conditioning regimen and prevent GvHD in mice [13, 34]. Using the Meth-A cell line (BALB/c-derived fibrosarcoma), we found that IBM-BMT plus the injection of $CD4^+$ T-cell-depleted (but not $CD8^+$ T cell depleted) spleen cells (as DLI) can prevent GvHD while suppressing tumor growth (Fig. 4). In addition, we have found that IBM-BMT plus extensive DLI (three times every 2 weeks) leads to the complete rejection of the tumor, although the success rate (three out of 50) is not high so far [34].

In addition, we have examined whether this strategy (IBM-BMT plus DLI) is applicable to other tumors in other animals. We have obtained similar results in another system (colon cancer, ACL-15 in rats) [35]. We are now establishing more efficient strategies to eradicate malignant tumors.

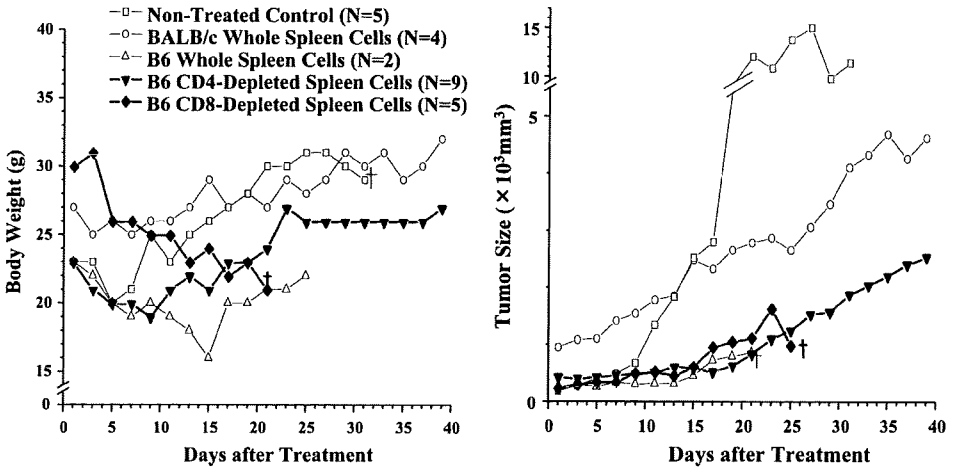

Fig. 4 Prevention of GvHD and suppression of tumor growth by IBM-BMT + DLI ($CD4^-$)

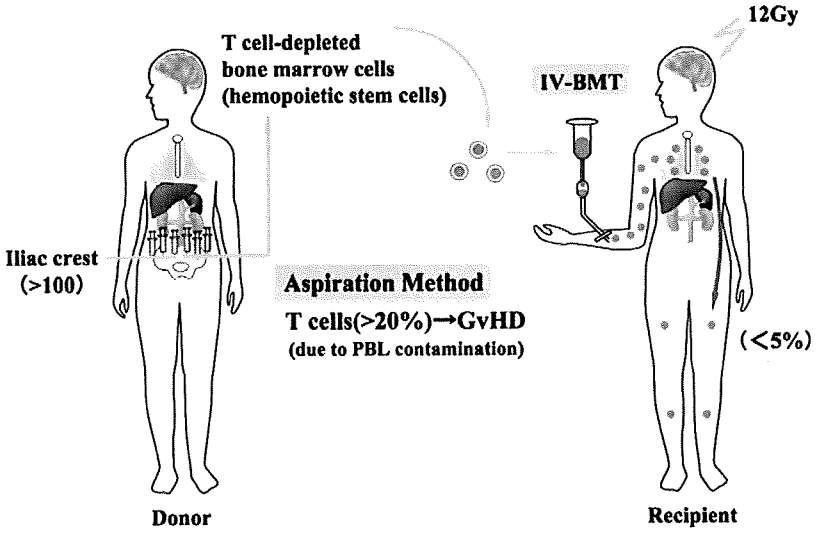

Fig. 5 Conventional BMT method for allogeneic BMT. Conventional BMT is carried out using an aspiration method (AM), followed by the intravenous injection of BMCs (IV-BMT)

Conventional BMT versus novel BMT

Conventional BMT is carried out as follows: bone marrow needles are inserted into the iliac bones more than 100 times, and the BMCs are collected by the aspiration method (AM; Fig. 5). Therefore, contamination with peripheral blood (particularly T cells) is inevitable. When thus-collected cells are intravenously injected, most cells become trapped in the lung and only a few cells migrate into the bone marrow (Fig. 5).

To apply our new BMT methods to humans, we established, using cynomolgus monkeys, a "perfusion method (PM)", which minimizes the contamination of BMCs with T cells. As shown in Fig. 6, two needles are inserted into a long bone such as the humerus, femur, or tibia. The end of the extension tube is connected to a needle. The other end is placed in a syringe containing 0.5 ml heparin. The second needle is connected to a syringe containing 30 ml of saline, and the saline is then pushed gently from the syringe into the medullary cavity to flush out the bone marrow. The saline containing the BM fluid is then collected (Fig. 6).

There is significantly less contamination with T cells when using the PM (<10%) than with the conventional AM (>20%). Therefore, T cell depletion is unnecessary with the PM, and whole BMCs can be used. However, in the case of the conventional AM, T cell depletion is necessary, and the loss of some important cells such as MSCs during the

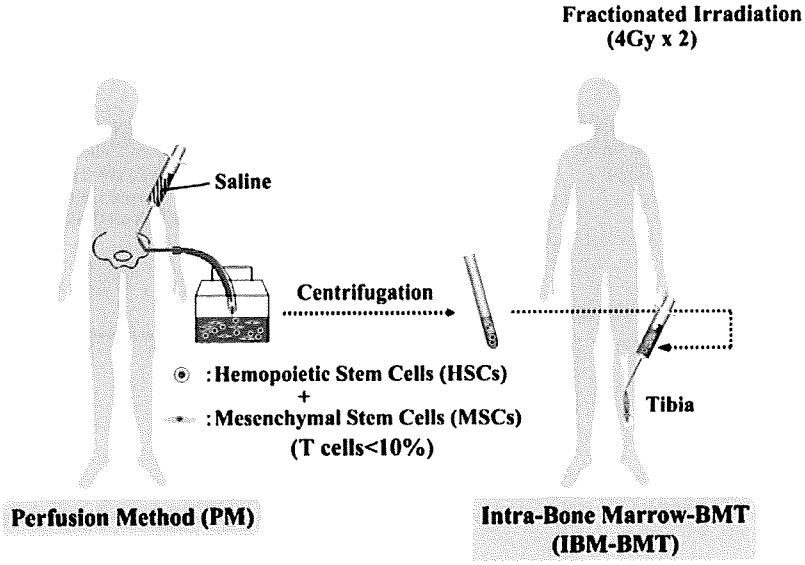

Fig. 6 New BMT method for allogeneic BMT. New BMT method is carried out using a perfusion method (*PM*), followed by IBM-BMT

process of T cell depletion is inevitable. Furthermore, the number and progenitor activities of the cells harvested using the PM are greater than when using the conventional AM [36, 37].

Future directions

We have also found that the PM is applicable to the iliac bones as well as the long bones not only in monkeys but also in humans.

As described here, the new BMT method (PM + IBM-BMT) can be used to treat various otherwise intractable diseases, including (1) autoimmune diseases, (2) age-associated diseases (osteoporosis, emphysema, etc.), (3) diseases curable by organ transplantation, and (4) malignant tumors (including solid tumors). The PM can efficiently be used to collect whole BMCs (including HSCs and MSCs) without them being contaminated with T cells, and no GvHD therefore develops. IBM-BMT can efficiently transfer donor whole BMCs (both HSCs and MSCs) into recipients and this method can therefore be used to quickly replace not only HSCs but also MSCs with donor-derived cells.

From the findings to date, it is conceivable that all the body's cells originate in the bone marrow, and that all diseases might therefore originate from defects in the bone marrow. One paper already suggests that gastric cancer originates from bone-marrow-derived cells [38].

We believe that the development of our BMT method heralds a revolution in the field of transplantation (BMT and organ transplantation) and regeneration therapy.

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LETTERS

edited by Jennifer Sills

China's Environmental Civilian Activism

IN THE POLICY FORUM "CHINA'S ROAD TO SUSTAINABILITY" (2 APRIL, P. 50), J. LIU OVERLOOKS AN important cultural force. China's worsening environmental conditions have catalyzed a spirit of environmental civilian activism.

For example, in 2003, a consortium proposed erecting 13 dams on the Nujiang River. China's environmental nongovernmental organizations and scholars launched a protest campaign through the Internet and newspapers. The critics argued that as reservoirs behind the dams filled up, flooding and landslides would imperil habitats. In response, Premier Wen Jiabao suspended the dam project pending an environmental review in 2004 (*1*).

The landmark of environmental civilian activism occurred in Xiamen City in 2007. The local government supported construction of a $1.4-billion paraxylene plant near the center of the city. Information about the environmental impact of this project was not made available to the local residents. The people of Xiamen City were outraged when—through cell phone messages and the Internet—they learned of the plant's environmental risks. A phone text message was circulated among Xiamen citizens in late May calling for a "collective walk" (demonstration). On 1 June 2007, more than 1000 citizens gathered in front of the municipal building to protest. The demonstration forced the local government to cancel the largest industrial project in the history of Xiamen (*2*).

Protests. Chinese citizens protest against the planned Guangzhou trash incinerator in 2009. Their banners read, "Oppose the trash incinerator."

The burgeoning middle class has become the driver of environmental civilian activism. For example, operation of the Likeng trash incinerator in Guangzhou City started in 2005 without any protest, although local farmers worried about health risk (*3*). In contrast, the proposed Panyu trash incinerator in Guangzhou City in 2009 triggered protests that were led by the middle class (*4*), who used science-based evidence to openly challenge prevailing notions formulated by the authorities. (In earlier years, standard practice was to obey Beijing-based experts in environmental protection.) In addition, the self-organized middle class forced the local government to open discussion by Internet. By seizing the opportunity for an open discussion, the newly empowered locals took to the streets to protect their environmental rights (*4*).

Recent years have witnessed an impressive growth in environmental protests in China. The number of petitions and mass public protests related to environmental issues has increased by 30% per year in the past few years, although the number of petitions lodged with the Chinese government has dropped (*5*).

The current environmental civilian activism movements have several common characteristics: (i) They are confined to one specific geographical space. (ii) Their goal is protecting the environment, rather than political rights or commercial interests. (iii) They focus on a specific pollutant, rather than general environmental degradation. The local nature of the movement enables the organization of a large number of citizens with little effort in a very short time. Given more open social and political conditions and the increasing size of the middle class in China, environmental civilian activism will certainly be a key driver in China's transition to sustainability.

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6. The views expressed in this paper are the author's own and not necessarily those of QIBEBT-CAS, GIG-CAS.

I thank B. Jong for comments and linguistic support.

Effects of China's Economic Growth

IN A RECENT POLICY FORUM, J. LIU REVIEWED "China's road to sustainability" (2 April, p. 50). Liu focused on population growth and an increase in the number of households, but he failed to adequately address the most important socioeconomic driver behind environmental degradation in China: rapid economic growth that is not offset by efficiency improvements (*1*, *2*).

In China, exports and capital investments contribute significantly more to gross domestic product (GDP) than household and government consumption combined (*3*), and this also holds true for emissions (*1*, *2*). From 2002 to 2005, the production of exports was responsible for 50% of the growth in carbon dioxide emissions and capital formation was responsible for 35%;

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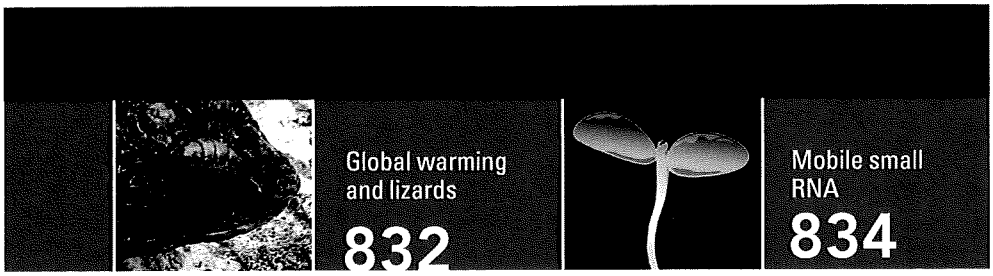

household and government consumption contributed 15% (*4*, *5*).

Liu discusses population control and household size, but a more dominant issue in terms of population dynamics is the migration from rural to urban areas (*6*). From 1990 to 2007, the urban population increased by 292 million, whereas the rural population decreased by 116 million (*3*). Urban dwellers, even if migrants from rural areas, have a higher income (*3*) and hence higher energy use and environmental impacts (*2*, *6*).

A key challenge for China is to continue strong economic growth while minimizing environmental impacts. Reductions in emissions per unit of GDP are unlikely to reduce total emissions if economic growth continues (*1*). China will need to combine aggressive domestic policies with international support to reverse the current growth in coal-dominated energy use and emissions.

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TECHNICAL COMMENT ABSTRACTS

Comment on "Detection of an Infectious Retrovirus, XMRV, in Blood Cells of Patients with Chronic Fatigue Syndrome"

Cathie Sudlow, Malcolm Macleod, Rustam Al-Shahi Salman, Jon Stone

Lombardi *et al.* (Reports, 23 October 2009, p. 585) reported an association between the human gammaretrovirus XMRV and chronic fatigue syndrome. However, their results may be misleading because of various potential sources of bias and confounding. If real, the association may lack generalizability because of the specific characteristics of the cases studied and could be due to reverse causality.

Full text at www.sciencemag.org/cgi/content/full/328/5980/825-a

Comment on "Detection of an Infectious Retrovirus, XMRV, in Blood Cells of Patients with Chronic Fatigue Syndrome"

Andrew Lloyd, Peter White, Simon Wessely, Michael Sharpe, Dedra Buchwald

Lombardi *et al.* (Reports, 23 October 2009, p. 585) reported a significant association between the human retrovirus XMRV and chronic fatigue syndrome (CFS). However, the cases with CFS and the control subjects in their study are poorly described and unlikely to be representative. Independent replication is a critical first step before accepting the validity of this finding.

Full text at www.sciencemag.org/cgi/content/full/328/5980/825-b

Comment on "Detection of an Infectious Retrovirus, XMRV, in Blood Cells of Patients with Chronic Fatigue Syndrome"

Jos W. M. van der Meer, Mihai G. Netea, Jochem M. D. Galama, Frank J. M. van Kuppeveld

Lombardi *et al.* (Reports, 23 October 2009, p. 585) reported detection of the human gammaretrovirus XMRV in the blood cells of patients with chronic fatigue syndrome (CFS). However, the patient description provided was incomplete. The inclusion of patients from a "CFS outbreak" previously linked with a viral infection, without confirmation in sporadic CFS cases, casts doubt on the role of XMRV in the pathogenesis of CFS.

Full text at www.sciencemag.org/cgi/content/full/328/5980/825-c

Response to Comments on "Detection of an Infectious Retrovirus, XMRV, in Blood Cells of Patients with Chronic Fatigue Syndrome"

Judy A. Mikovits and Francis W. Ruscetti

We reported the detection of the human gammaretrovirus XMRV in 67% of 101 patients with chronic fatigue syndrome (CFS) and in 3.7% of 218 healthy controls, but we did not claim that XMRV causes CFS. Here, we explain why the criticisms of Sudlow *et al.*, Lloyd *et al.*, and van der Meer *et al.* regarding the selection of patients and controls in our study are unwarranted.

Full text at www.sciencemag.org/cgi/content/full/328/5980/825-d

Risks of Immune System Treatments

WE WISH TO ADD SEVERAL POINTS TO THE News Focus story "Replacing an immune system gone haywire" (J. Couzin-Frankel, 12 February, p. 772).

First, a great deal of research had already been done before the 1996 Basel meeting mentioned in the story. Stem cell transplants had been studied in animal models of autoimmune disease (*1*–*5*). Patient stem cell transplant protocols had been written, and a few human patients had already been treated specifically for autoimmune disease (*6*–*10*).

Second, we would like to stress the varying levels of risk in the treatment strategies described in the story. The immune system originates from hematopoietic stem cells (HSCs). Before receiving a transplant, patients with autoimmune diseases receive "conditioning" chemotherapy or radiation that destroys lymphocytes, inducing an immediate immune cease-fire. Subsequently, HSCs are infused to regenerate a new self-tolerant immune system. Sullivan and Nash advocate conditioning regimens with high doses of radiation. These extreme regimens cause irreversible bone marrow failure, thus requiring mandatory HSC reinfusion. The rationale for this high-dose strategy is that maximal ablation of the immune system will translate into longer and more durable disease remission. In contrast, we advocate less extreme regimens of chemotherapy, which can halt inflammation without altering the bone marrow's ability to recover. The News Focus article also comments on the risk of infertility when patients are pretreated with chemotherapy. We emphasize that the risk of infertility is higher for the more extreme regimens.

Finally, although the News Focus story comments on problems obtaining insurance approval in the United States, medical funding is a worldwide issue, including in countries with government-funded health services. In addition to patient safety benefits, less toxic regimens also cost any health care system less money, because patients are less likely to suffer complications such as secondary cancers.

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CORRECTIONS AND CLARIFICATIONS

Letters: "Climate change and the integrity of science" by P. H. Gleick *et al.* (7 May, p. 689). Due to an editorial error, the original image was not a photograph but a collage. It was a mistake to have used it. The image (link available at www.sciencemag.org/cgi/content/full/328/5979/689/DC2) has been replaced in the HTML version and in the online PDF by an unaltered photograph from National Geographic (CREDIT: Paul Nicklen/National Geographic/Getty Images) of two polar bears on an ice floe.

News Focus: "Meeting briefs: The ins and outs of HIV" by J. Cohen (5 March, p. 1196). The earliest report of HIV predominantly entering cells through endocytosis appeared in C. D. Pauza, T. M. Price, *J. Cell Biol.* **107**, 959 (1988).

Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the previous 3 months or issues of general interest. They can be submitted through the Web (www.submit2science.org) or by regular mail (1200 New York Ave., NW, Washington, DC 20005, USA). Letters are not acknowledged upon receipt, nor are authors generally consulted before publication. Whether published in full or in part, letters are subject to editing for clarity and space.

HapMap scanning of novel human minor histocompatibility antigens

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Minor histocompatibility antigens (mHags) are molecular targets of allo-immunity associated with hematopoietic stem cell transplantation (HSCT) and involved in graft-versus-host disease, but they also have beneficial antitumor activity. mHags are typically defined by host SNPs that are not shared by the donor and are immunologically recognized by cytotoxic T cells isolated from post-HSCT patients. However, the number of molecularly identified mHags is still too small to allow prospective studies of their clinical importance in transplantation medicine, mostly due to the lack of an efficient method for isolation. Here we show that when combined with conventional immunologic assays, the large data set from the International HapMap Project can be directly used for genetic mapping of novel mHags. Based on the immunologically determined mHag status in HapMap panels, a target mHag locus can be uniquely mapped through whole genome association scanning taking advantage of the unprecedented resolution and power obtained with more than 3 000 000 markers. The feasibility of our approach could be supported by extensive simulations and further confirmed by actually isolating 2 novel mHags as well as 1 previously identified example. The HapMap data set represents an invaluable resource for investigating human variation, with obvious applications in genetic mapping of clinically relevant human traits. (Blood. 2009;113:5041-5048)

Introduction

The antitumor activity of allogeneic hematopoietic stem cell transplantation (HSCT), which is a curative treatment for many patients with hematologic malignancies, is mediated in part by immune responses that are elicited as a consequence of incompatibility in genetic polymorphisms between the donor and the recipient.[1,2] Analysis of patients treated for posttransplantation relapse with donor lymphocytes has shown tumor regression to be correlated with expansion of cytotoxic T lymphocytes (CTLs) specific for hematopoiesis-restricted minor histocompatibility antigens (mHags).[3,4] mHags are peptides, presented by major histocompatibility complex (MHC) molecules, derived from intracellular proteins that differ between donor and recipient due mostly to single nucleotide polymorphisms (SNPs) or copy number variations (CNVs).[1,2,5] Identification and characterization of mHags that are specifically expressed in hematopoietic but not in other normal tissues could contribute to graft-versus-leukemia/lymphoma (GVL) effects, while minimizing unfavorable graft-versus-host disease, one of the most serious complications of allo-HSCT.[1,2] Unfortunately, however, efforts to prospectively target mHags to invoke T cell–mediated selective GVL effects have been hampered by the scarcity of eligible mHags, largely due to the lack of efficient methods for mapping the relevant genetic loci. Several methods have been developed to identify mHags, including peptide elution from MHC,[6,7] cDNA expression cloning,[8,9] and linkage analysis.[3,10] We have recently reported a novel genetic method that combines whole genome association scanning with conventional chromium release cytotoxicity assays (CRAs). With this approach the genetic loci of the mHag gene recognized by a given CTL clone can be precisely identified using SNP array analysis of pooled DNA generated from immortalized lymphoblastoid cell lines (LCLs) that are immunophenotyped into $mHag^+$ and $mHag^-$ groups by CRA.[11] The mapping resolution has now been improved from several Mb for conventional linkage analysis to an average haplotype block size of less than 100 kb,[12] usually containing a handful of candidate genes. Nevertheless, it still requires laborious DNA pooling and scanning of SNP arrays with professional expertise for individual

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*M.K. and Y.N. are first coauthors and contributed equally to this work.

†S.O. and Y.A. are senior coauthors.

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

The online version of this article contains a data supplement.

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CTLs.[11] To circumvent these drawbacks, we have sought to take advantage of publicly available HapMap resources. Here, we describe a powerful approach for rapidly identifying mHAg loci using a large genotyping data set and LCLs from the International HapMap Project for genome-wide association analysis.[13-15]

Methods

Cell lines and CTL clones

The HapMap LCL samples were purchased from the Coriell Institute (Camden, NJ). All LCLs were maintained in RPMI1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1 mM sodium pyruvate. Because the recognition of a mHag requires presentation on a particular type of HLA molecule, the LCLs were stably transduced with a retroviral vector encoding the restriction HLA cDNA for a given CTL clone when necessary.[16]

CTL lines were generated from recipient peripheral blood mononuclear cells obtained after transplantation by stimulation with those harvested before HSCT after irradiation (33 Gy), and thereafter stimulated weekly in RPMI 1640 supplemented with 10% pooled human serum and 2 mM L-glutamine. Recombinant human inerleukin-2 was added on days 1 and 5 after the second and third stimulations. CTL clones were isolated by standard limiting dilution and expanded as previously described.[10,17] HLA restriction was determined by conventional CRAs against a panel of LCLs sharing HLA alleles with the CTLs. All clinical samples were collected based on a protocol approved by the Institutional Review Board Committee at Aichi Cancer Center and the University of Tokyoand after written informed consent was obtained in accordance with the Declaration of Helsinki.

Immunophenotyping of HapMap LCLs and high-density genome-wide scanning of mHag loci

Case ($mHag^+$) - control ($mHag^-$) LCL panels were generated by screening corresponding restriction HLA-transduced CHB and JPT HapMap LCL panels with each CTL clone using CRAs. Briefly, target cells were labeled with 0.1 mCi of ^{51}Cr for 2 hours, and 10^3 target cells per well were mixed with CTL at a predetermined E/T ratio in a standard 4-hour CRA. All assays were performed at least in duplicate. The percent specific lysis was calculated by ((Experimental cpm - Spontaneous cpm) / (Maximum cpm - Spontaneous cpm)) × 100. After normalization by dividing their percent specific lysis values by that of positive control LCL (typically recipient-derived LCL corresponding to individual CTL clones), the mHag status of each HapMap LCL was defined as positive, negative, or undetermined.

To identify mHag loci, we performed association tests for all the Phase II HapMap SNPs, by calculating χ^2 test statistics based on 2 × 2 contingency tables with regard to the mHag status as measured by CRA and the HapMap genotypes (presence or absence of a particular allele) at each locus. χ^2 were calculated for the 2 possible mHag alleles at each locus and the larger value was adopted for each SNP. While different test statistics may be used showing different performance, the χ^2 statistic is most convenient for the purpose of power estimation as described below. The maximum value of the χ^2 statistics was evaluated against the thresholds empirically calculated from 100 000 random permutations within a given LCL set. The program was written in C++ and will run on a unix clone. It will be freely distributed on request. Computation of the statistics was performed within several seconds on a Macintosh equipped with 2 × quadcore 3.2 GHz Zeon processors (Apple, Cupertino, CA), although 100 000 permutations took several hours on average.

Evaluation of the power of association tests using HapMap samples

The genotyping data of the Phase II HapMap[14] were obtained from the International HapMap Project website (http://www.hapmap.org/genotypes/latest_ncbi_build35), among which we used the nonredundant data sets (excluding SNPs on the Y chromosome) from 60 CEU (Utah residents with ancestry from northern and western Europe) parents, 60 YRI (Yoruba in Ibadan, Nigeria) parents, and the combined set of 45 JPT (Japanese in Tokyo, Japan) and 45 CHB (Han Chinese in Beijing, China) unrelated people. They contained 3 901 416 (2 624 947 polymorphic), 3 843 537 (295 293 polymorphic), and 3 933 720 (2 516 310 polymorphic) SNPs for CEU, YRI, and JPT + CHB, respectively.

To evaluate the power, we first assumed that the Phase II HapMap SNP set contains the target SNP of the relevant mHag or its complete proxies, and that the immunologic assays can completely discriminate i $mHag^+$ and j $mHag^-$ HapMap LCLs. Under this ideal condition, the test statistic, or χ^2, for these SNPs takes a definite value, $f(i,j) = i+j$, which was compared with the maximum χ^2 value, or its distribution, under the null hypothesis, that is, no SNPs within the Phase II HapMap set should be associated with the mHag locus. Unfortunately, the latter distribution cannot be calculated in an explicit analytical form but needs to be empirically determined based on HapMap data, because Phase II HapMap SNPs are mutually interdependent due to extensive linkage disequilibrium within human populations. For this purpose, we simulated 10 000 case-control panels by randomly choosing i $mHag^+$ and j $mHag^-$ HapMap LCLs for various combinations of (i,j) and calculated the maximum χ^2 values (χ^2_{max}) for each panel to identify those (i,j) combinations, in which $f(i,j)$ exceeds the upper 1 percentile point of the simulated 10 000 maximum values, $g(i,j)^{P=.01}$.

When proxies are not complete (ie, $r^2 < 1$), the expected values will be decayed by the factor of r^2, and further reduced due to the probabilities of false positive (f_P) and negative (f_N) assays, and expressed as $\hat{f}(i,j) = (i+j) \times f^2$ through an apparent r^2 (f^2) as provided in formula 1.[1] Under given probabilities of assay errors and maximum LD strength between markers and the mHag allele, we can expect to identify target mHag loci for those (i,j) sets that satisfy $\hat{f}(i,j) > g(i,j)^{P=.01}$.

Empirical estimation of distributions of r^2

The maximum r^2 value (r^2_{max}) between a given mHag allele and one or more Phase II HapMap SNPs was estimated based on the observed HapMap data set. Each Phase II HapMap SNP was assumed to represent a target mHag allele, and the (r^2_{max}) was calculated, taking into account all the Phase II HapMap SNPs less than 500 kb apart from the target SNP.

Confirmatory genotyping

Genotyping was carried out either by TaqMan MGB technology (Applied Biosystems, Foster City, CA) with primers and probes for HA-1 mHag according to the manufacturer's protocol using an ABI 7900HT with the aid of SDS version 2.2 software (Applied Biosystems) or by direct sequencing of amplified cDNA for the *SLC1A5* gene. cDNA was reverse transcribed from total RNA extracted from LCLs, and polymerase chain reaction (PCR) was conducted with cDNA with the corresponding primers. Amplified DNA samples were sequenced using BigDye Terminator version 3.1 (Applied Biosystems). The presence or absence (deletion) of the *UGT2B17* gene was confirmed by genomic PCR with 2 primer sets for exons 1 and 6 as described previously[18] using DNA isolated from LCLs of interest.

Epitope mapping

A series of deletion mutant cDNAs were designed and cloned into pcDNA3.1/V5-His TOPO plasmid (Invitrogen, Carlsbad, CA). Thereafter, 293T cells that had been transduced with restricting HLA class I cDNA for individual CTL clones were transfected with each of the deletion mutants and cocultured with the CTL clone overnight to induce interferon (IFN)-γ release, which was then evaluated by enzyme-linked immunosorbent assay (ELISA) as previously described.[9]

For *SLC1A5*, expression plasmids encoding full-length cDNA and the exon 1 of recipient and donor origin were first constructed because only the SNP in the exon 1 was found to be concordant with susceptibility to CTL-3B6. Next, amino (N)- and (carboxyl) C-terminus–truncated mini-genes encoding polypeptides around the polymorphic amino acid defined by the SNP were amplified by PCR from *SLC1A5* exon 1 cDNA as template and cloned into the above plasmid. The constructs all encoded a Kozak

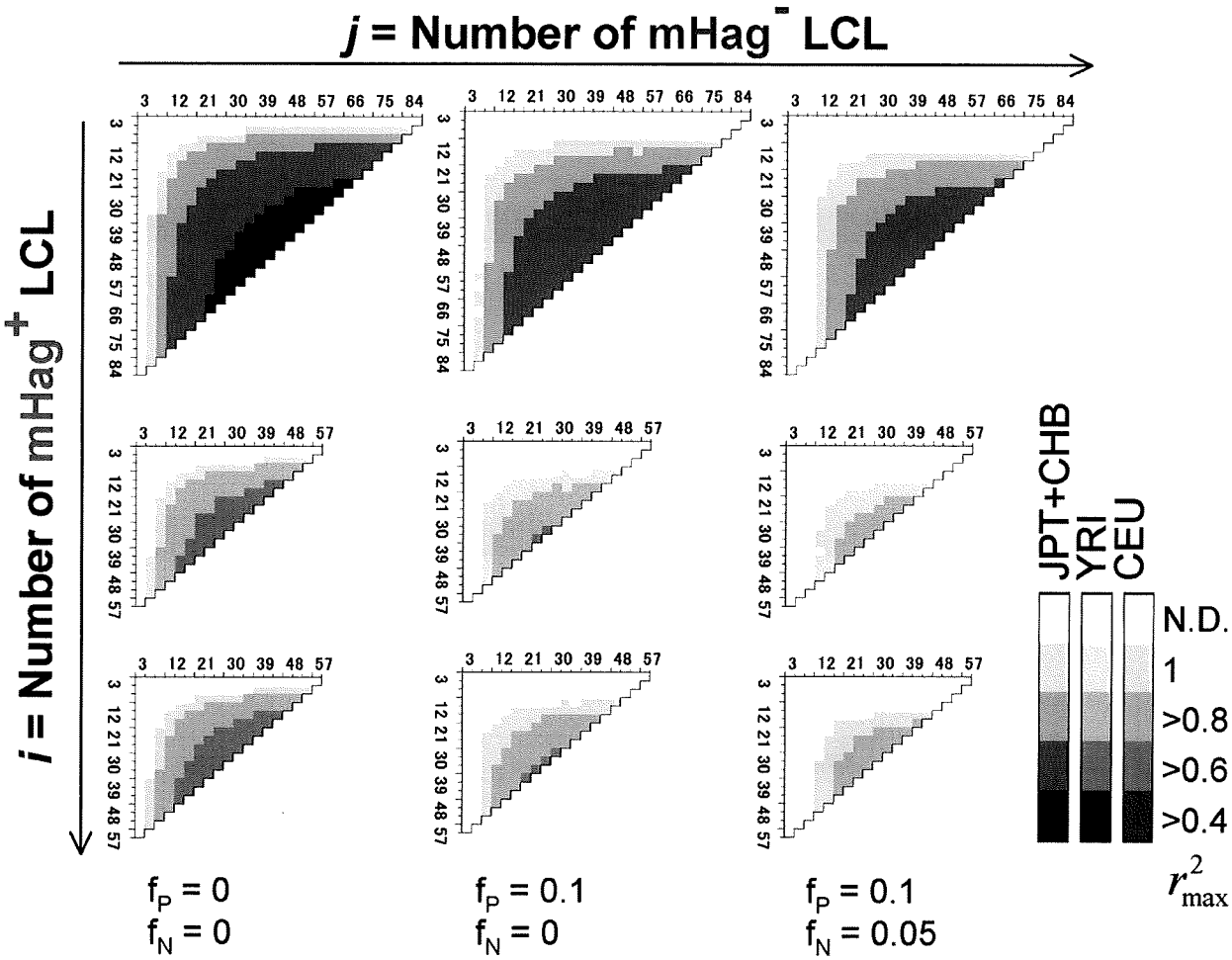

Figure 1. Numbers of positive and negative LCLs required for successful mHag mapping. The target locus was assumed to be uniquely identified, if the expected χ^2 value for the target SNP ($\hat{\chi}(i,j)$, see Document S1) exceeded the upper 1 percentile point of the maximum χ^2 values in 10 000 simulated case-control panels ($g(i,j)^{P=.01}$). Combinations of the numbers of mHag$^+$ (vertical coordinates) and mHag$^-$ (horizontal coordinates) samples satisfying the above condition are shown in color gradients corresponding to different max r^2 values between the target SNP and one or more nearby Phase II HapMap SNPs (r^2_{max}), ranging from 0.4 to 1.0. Calculations were made for 3 HapMap population panels, CHB + JPT (top), YRI (middle), and CEU (bottom) and for different false positive and negative rates, $f_P = f_N = 0$ (left), $f_P = 0.1, f_N \cong 0$ (middle), and $f_P = 0.1, f_N = 0.05$ (right), considering the very low false negative assays for CRAs.

sequence and initiator methionine (CCACC-ATG) and for C-terminus deletions a stop codon (TAG).

For *UGT2B17*, a series of C-terminus deletion mutants with approximately 200 bp spacing was first constructed as above. For further mapping, N-terminus deletion mutants were added to the region that was deduced to be potentially encoding the CTL-1B2 epitope. For prediction of a CTL epitope, the HLA Peptide Binding Predictions algorithm on the BioInformatics & Molecular Analysis Section (BIMAS) website (http://www.bimas.cit.nih.gov/molbio/hla_bind/)[19] was used because HLA-A*0206 has a similar binding motif to that of A*0201.

Epitope reconstitution assay

The candidate mHag epitopes and allelic counterpart peptides (in case of SLC1A5) were synthesized by standard Fmoc chemistry. ^{51}Cr-labeled mHag$^-$ donor LCL were incubated with graded concentrations of the peptides and then used as targets in standard CRAs.

Results and discussion

Statistical approach and estimation of potential overfitting

We reasoned that the mHag locus recognized by a given CTL clone could be defined by grouping LCLs from a HapMap panel into mHag$^+$ and mHag$^-$ subpanels according to their susceptibility to lysis by the CTL clone and then performing an association scan using the highly qualified HapMap data set containing more than 3 000 000 SNP markers. The relevant genetic trait here is expected to show near-complete penetrance, and the major concern with this approach arises from the risk of overfitting observed phenotypes to one or more incidental SNPs with this large number of HapMap SNPs under the relatively limited size of freedom due to small numbers of independent HapMap samples (90 for JPT + CHB and 60 for CEU and YRI, when not including their offspring).[13]

To address this problem, we first estimated the maximum sizes of the test statistics (here, χ^2 values) under the null hypothesis (ie, no associated SNPs within the HapMap set) by simulating 10 000 case-control HapMap panels under different experimental conditions, and compared them with the expected size of test statistic values from the marker SNPs associated with the target SNP, assuming different linkage disequilibrium (LD), or r^2 values in between. As shown in Figure 1, the possibility of overfitting became progressively reduced as the number of LCLs increased, which would allow for identification of the target locus in a broad range of r^2 values, except for those mHags having very low minor allele frequencies (MAF) below

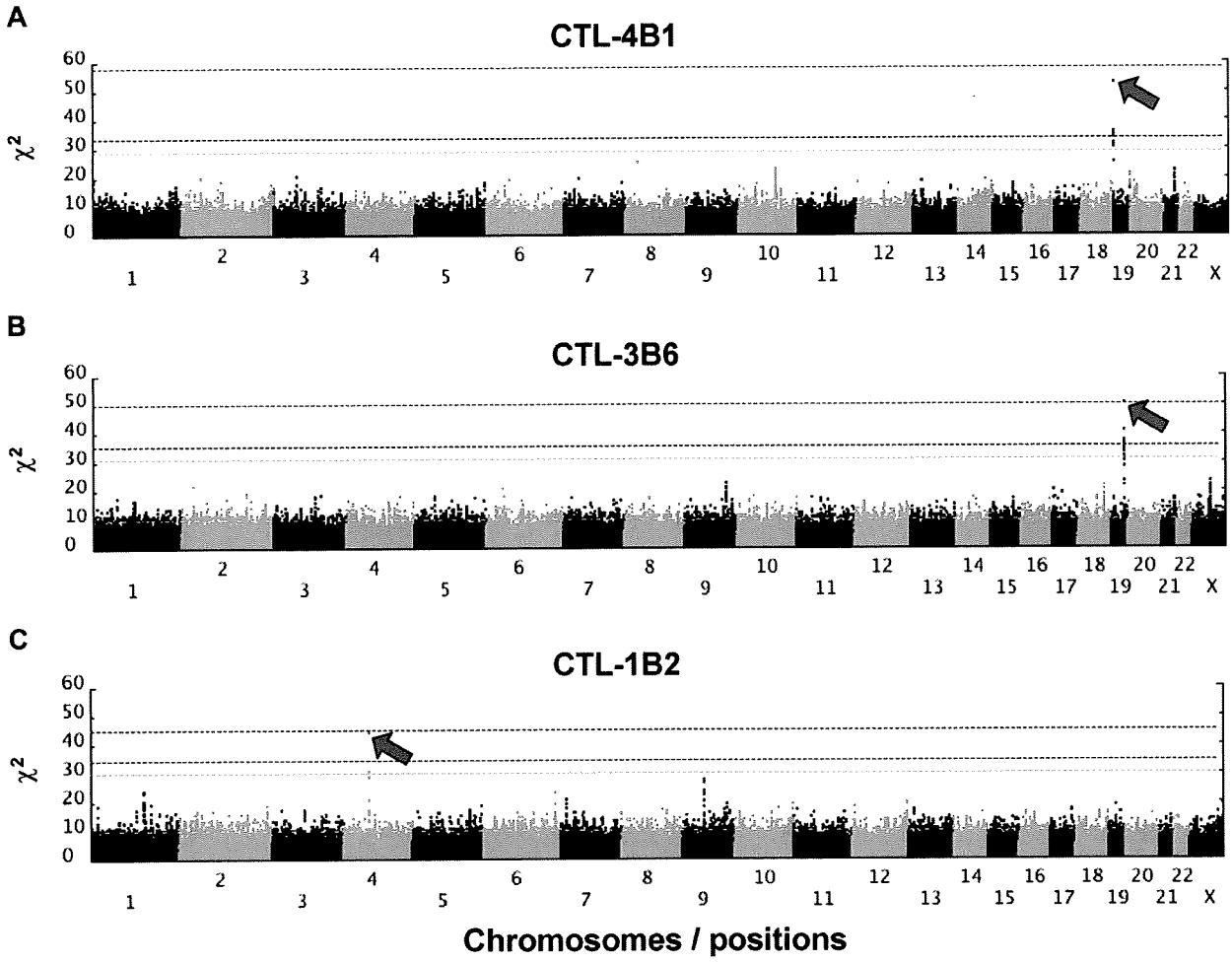

Figure 2. Genome-wide scanning to identify chromosome location of mHag. χ^2 values were plotted against positions on each chromosome for each of 3 mHags recognized by CTL-4B1 (A), CTL-3B6 (B), and CTL-1B2 (C). Chromosomes are displayed in alternating colors. Threshold χ^2 values corresponding to the genome-wide $P = 10^{-3}$ (dark blue) and 10^{-2} (light blue), as empirically determined from 100 000 random permutations, are indicated by broken lines, while the theoretically possible maximum values are shown with red broken lines. The highest χ^2 value in each experiment is indicated by a red arrow.

approximately 0.05. According to our estimation using the Phase II HapMap data (see "Methods"), the majority (> 90%) of common target SNPs (MAF > ~ 0.05) could be captured by one or more HapMap SNPs with more than 0.8 of r^2 (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), ensuring a high probability of detecting an association (Figure 1 left panels). The simulation of pseudo-Phase II sets generated from the ENCODE regions provided a similar estimation.[13] False positive and negative immunophenotyping results could also complicate the detection, reducing the expected test statistics through the "apparent" r^2 values ($\hat{r}^2$), as defined by

$$\hat{r}^2 = r^2 \times \frac{(1 - f_P - f_N)^2}{(1 - f_P + f_N q)(1 - f_N + f_P/q)} \quad (1)$$

where f_P, f_N, and q represent false typing probabilities with positive and negative LCL panels, and the ratio of the positive to the negative LCL number, respectively. However, the high precision of cytotoxicity assays (f_P ~ < 0.1, f_N ~ = 0) limits this drawback from the second term to within acceptable levels and allows for sensitive mHag locus mapping with practical sample sizes (Figure 1 middle and right panels), suggesting the robustness of our novel approach.

Evaluation of the detection power for known mHags

Based on these considerations, we then assessed whether this approach could be used to correctly pinpoint known mHag loci (Table S1). Because the relevant mHag alleles are common SNPs and directly genotyped in the Phase II HapMap set, or if not, located within a well-defined LD block recognized in this set (Figure S2), their loci would be expected to be uniquely determined with an acceptable number of samples, as predicted from Figure 1. To test this experimentally, we first mapped the locus for HA-1^H mHag[7] by evaluating recognition of the HLA-A*0206–transduced HapMap cell panel with HLA-A*0206–restricted CTL-4B1.[20] After screening 58 well-growing LCLs from the JPT + CHB panel with CRAs using CTL-4B1 (Figure S3A; Tables S2,S3), we obtained 37 $mHag^+$ and 21 $mHag^-$ LCLs, which were tested for association at 3 933 720 SNP loci. The SNP (rs1801284) encoding the mHag is located within a HapMap LD block on chromosome 19q13.3, but is not directly genotyped within this data set. The genome-wide scan clearly indicated a unique association with the HA-1^H locus within the *HMHA1* gene, showing a peak χ^2 statistic of 52.8 (not reached in 100 000 permutations) at rs10421359 (Figures 2A,3A; Tables S2,S3).

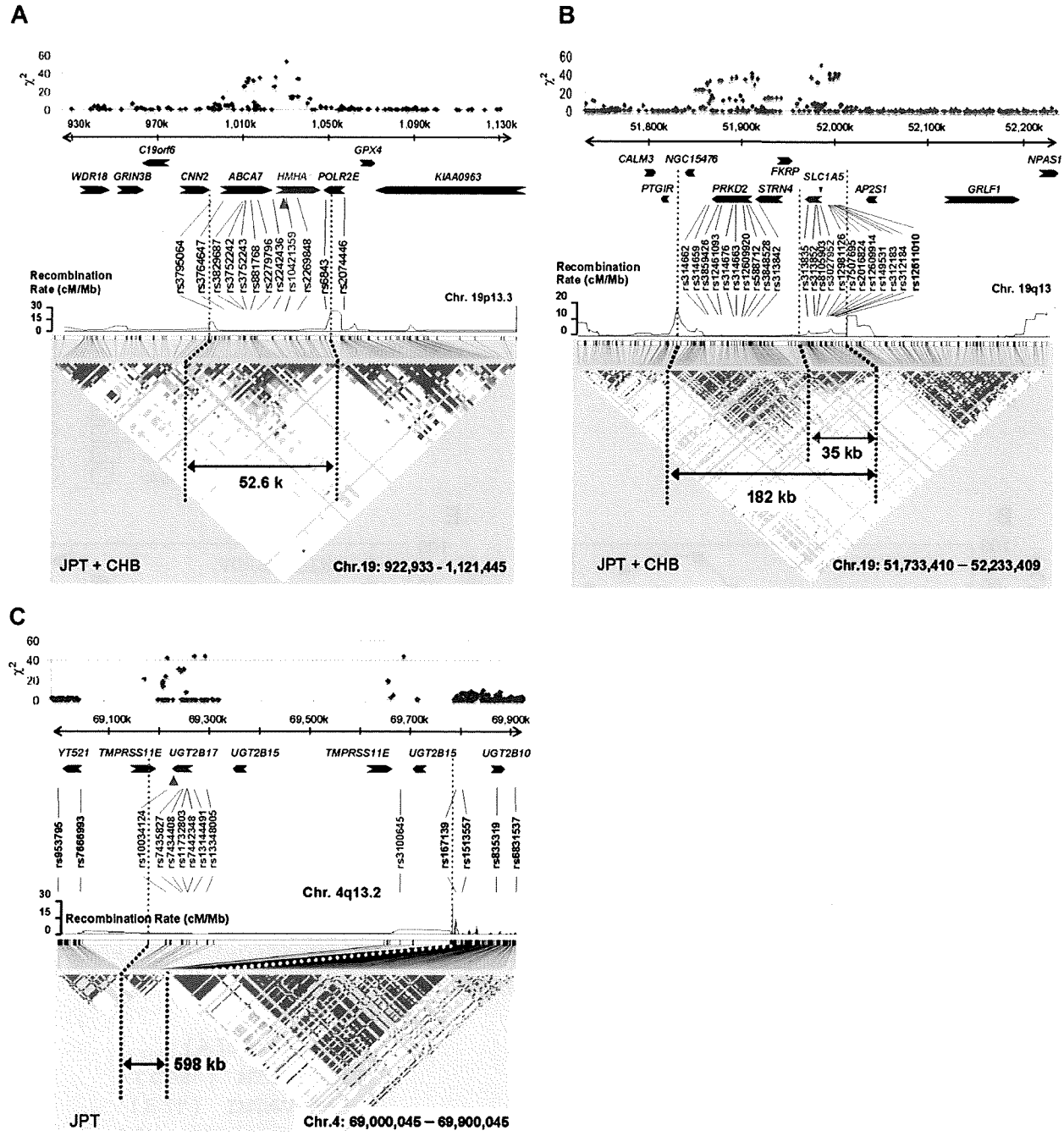

Figure 3. Regions of mHag loci identified by HapMap scanning. LD structures around the SNPs showing peak statistical values (in JPT + CHB) are presented for each mHag locus identified with (A) CTL-4B1, (B) CTL-3B6, and (C) CTL-1B2. Regional χ^2 plots are also provided on the top of each panel. LD plots in pairwise D's with recombination rates along the segment were drawn with HaploView software version 4.0 (http://www.broad.mit.edu/mpg/haploview/). The size and location of each LD block containing a mHag locus are indicated within the panels. Significant SNPs (blue letters), as well as other representative SNPs, are shown in relation to known genes. The positions of the SNPs showing the highest statistic values (red letters) are indicated by red arrowheads.

Identification of novel mHags

We next applied this method to mapping novel mHags recognized by CTL clone 3B6, which is HLA-B*4002–restricted; and CTL clone 1B2, which is HLA-A*0206–restricted. Both clones had been isolated from peripheral blood samples of post-HSCT different patients. In preliminary CRAs with the JPT + CHB panel, allele frequencies of target mHags for CTL-3B6 and CTL-1B2 in this panel were estimated as approximately 25% and approximately 45%, respectively (data not shown). After screening 72 JPT + CHB LCLs with CTL-3B6, 36 mHag$^+$ and 14 mHag$^-$ LCLs were obtained, leaving 22 LCLs undetermined based on empirically determined thresholds ($> 51\%$ for mHag + LCLs and $< 11\%$ for mHag-LCLs; Figure S3B, Tables S2,S4). As shown in Figure 2B, the χ^2 statistics calculated from the immunophenotyping data produced discrete peaks in the LCL sets. The peak in chromosome 19q13.3 for the CTL-3B6 set showed the theoretically maximum χ^2 value of 50 (not reached in 100 000 permutations) at rs3027952, which was mapped within a small LD block of

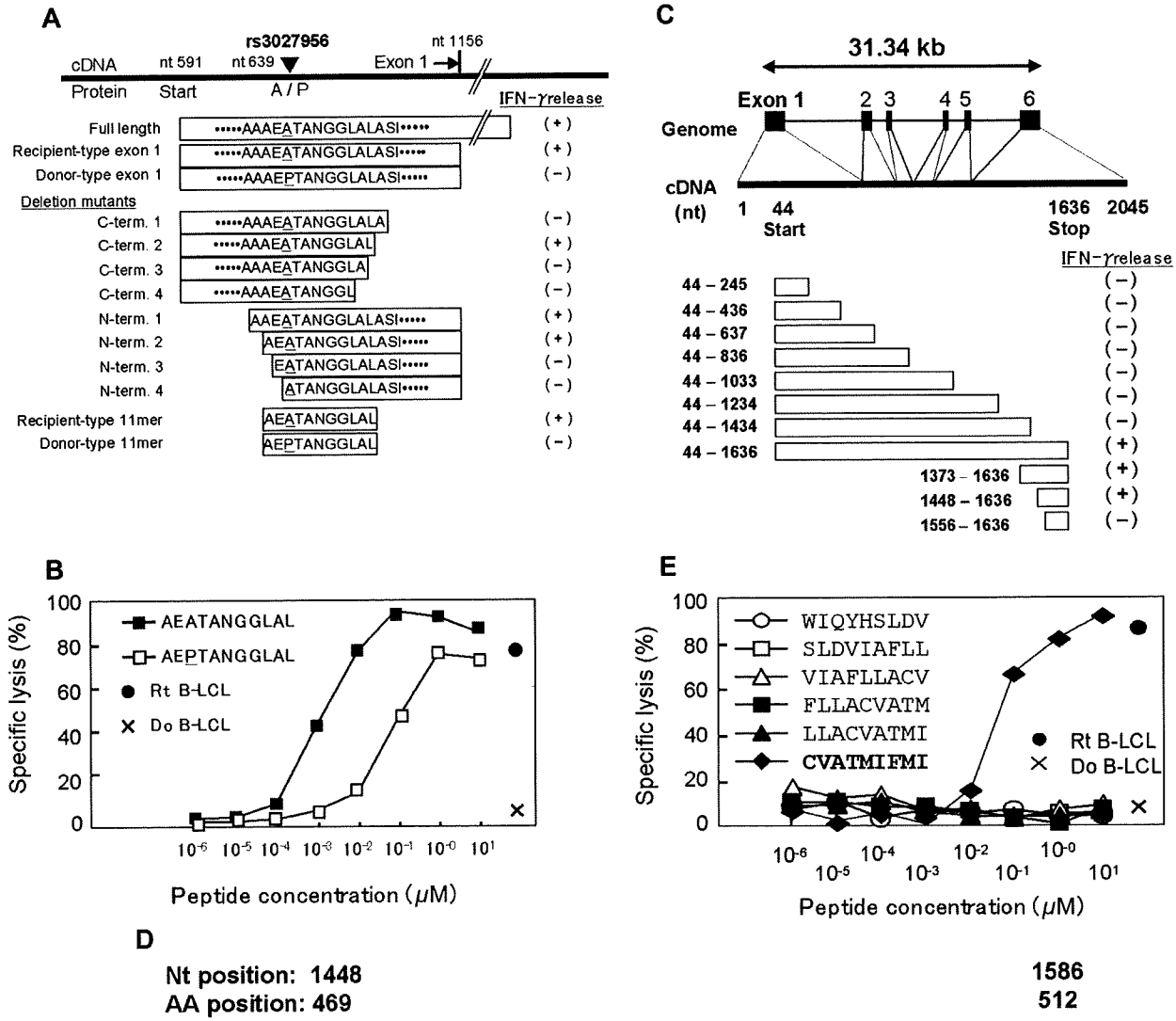

Figure 4. Epitope mapping. (A) Determination of the *SLC1A5* epitope by deletion mapping. Plasmids encoding recipient full-length *SLC1A5*, exon 1 of recipient and donor, exon 1 with various N- and C-terminus deletions around the amino acid encoded by SNP rs51983014, and minigenes encoding AEATANGGLAL and its allelic counterpart AEPTANGGLAL were constructed and transfected into HLA-B*4002–transduced 293T cells. Interferon (IFN)-γ was assessed by ELISA (right column) after coculture of CTL-3B1 with 293T transfectants. (B) Epitope reconstitution assay with synthetic undecameric peptides, AEATANGGLAL and AEPTANGGLAL. (C) Structure of the *UGT2B17* gene and screening of *UGT2B17* cDNA and deletion mutants. HLA-A*0206–transduced 293T cells were transfected with each plasmid and cocultured with CTL-2B1. IFN-γ production from CTL-1B2 (right column) indicated that the epitope was likely encoded by nucleotides 1448-1586, including 30 nucleotides from position 1566 that could potentially encode part of the epitope. (D) Epitope prediction using the HLA Peptide Binding Predictions algorithm.[19] Because HLA-A*0201 and -A*0206 have similar peptide binding motifs,[30] the algorithm for HLA-A*0201 was used to predict candidate epitopes recognized by CTL-1B2. Values in parentheses indicate the predicted half-time of dissociation. (E) Epitope reconstitution assays with graded concentrations of synthetic nonameric peptides shown in panel D.

approximately 182 kb, or more narrowly within its 35 kb sub-block containing a single gene, *SLC1A5*, as a candidate mHag gene (Figure 3B). In fact, when expressed in 293T cells with HLA-B*4002 transgene, recipient-derived, but not donor-derived, *SLC1A5* cDNA was able to stimulate IFN-γ secretion from CTL-3B6 (Figure 4A), indicating that *SLC1A5* actually encodes the target mHag recognized by CTL-3B6. Conventional epitope mapping using a series of deletion mutants of *SLC1A5* cDNA finally identified an undecameric peptide, AEATANGGLAL, as the minimal epitope (Figure 4A). The donor-type AEPTANGGLAL induced IFN-γ with a 2-log lower efficiency, suggesting that AEPTANGGLAL may not be transported efficiently into the ER

because endogenous expression of a minigene encoding AEPTANGGLAL was not recognized by CTL-3B1 (Figure 4B). Unfortunately, although the peak statistic value showed the theoretically maximum value for this data set, it did not conform to the relevant SNP for this mHag (rs3027956) due to high genotyping errors of the HapMap data at this particular SNP. However, the result of our resequencing showed complete concordance with the presence of the rs3027956 SNP and recognition in the cytotoxicity assay (Table S4).

Similarly, 13 mHag$^+$ and 32 mHag$^-$ LCLs were identified from the screening of 45 JPT LCLs from the same panel using CTL-1B2 (Figure S3C; Tables S2,S5). The χ^2 statistics calculated from the immunophenotyping data produced bimodal discrete peaks with this LCL set. The target locus for the mHag recognized by CTL-1B2 was identified at a peak (max $\chi^2 = 44$, not reached in 100 000 permutations) within a 598-kb block on chromosome 4q13.1, coinciding with the locus for a previously reported mHag, *UGT2B17*[18] (Figures 2C, 3C). In fact, our epitope mapping using *UGT2B17* cDNA deletion mutants (Figure 4C), prediction of candidate epitopes by HLA-binding algorithms[19] (Figure 4D) and epitope reconstitution assays (Figure 4E), successfully identified a novel nonameric peptide, CVATMIFMI. Of particular note, this mHag was not defined by a SNP but by a CNV (ie, a null allele[18]) that is in complete LD with the SNPs showing the maximum χ^2 value (Table S5). Transplanted T cells from donors lacking both *UGT2B17* alleles are sensitized in recipients possessing at least 1 copy of this gene.[18] Although LD between SNPs and CNVs has been reported to be less prominent,[21] this is an example where a CNV trait could be captured by a SNP-based genome-wide association study.

The recent generation of the HapMap has had a profound impact on human genetics.[13,15] In the field of medical genetics, the HapMap is a central resource for the development of theories and methods that have made well-powered, genome-wide association studies of common human diseases a reality.[22-28] The HapMap samples provide not only an invaluable reference for genetic variations within human populations, but highly qualified genotypes that enable gene-wide scanning. Here, we have demonstrated how effectively HapMap resources can be used for genetic mapping of clinically relevant human traits. No imputations and tagging strategies are required[25,28] and the potential loss of statistical power due to very limited sample sizes is circumvented by accurate immunologic detection of the traits.

Using publicly available HapMap resources, high-throughput identification of mHag genes is possible without highly specialized equipment or expensive microarrays. Except for clinically irrelevant mHags with very low allele frequencies (eg, MAF $< 5\%$), the target of a given CTL can be sensitively mapped within a mean LD block size, typically containing just a few candidate genes. The methodology described here will facilitate construction of a large panel of human mHags including those presented by MHC class II molecules, and promote our understanding of human allo-immunity and development of targeted allo-immune therapies for hematologic malignancies.[1,2] The HapMap scan approach may be useful for exploring other genetic traits or molecular targets (eg, differential responses to some stress or drugs), if they can be discriminated accurately through appropriate biologic assays. In this context, the recent report that we may reprogram the fate of terminally differentiated human cells[29] is encouraging, indicating possible exploration of genotypes that are relevant to cell types other than immortalized B cells.

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Authorship

Contribution: M.K. performed most of immunologic experiments and analyzed data and wrote the manuscript; Y.N. performed the majority of genetic analyses and analyzed the data; H.T., T.K., M.Y., S.M. and K.Tsujimura performed research; K.Taura contributed to the computational simulation; Y.I., Taro T., K.M., Y.K. and Y.M. collected clinical data and specimens; T.I., H.T., S.R.R., Toshitada T. and K.K. contributed to data analysis and interpretation, and writing of the article; and Y.A. and S.O. supervised the entire project, designed and coordinated most of the experiments in this study, and contributed to manuscript preparation.

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ORIGINAL ARTICLE

Aberrant expression of BCL2A1-restricted minor histocompatibility antigens in melanoma cells: application for allogeneic transplantation

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Abstract It has been shown that allogeneic hematopoietic stem cell transplantation (HSCT) can be one of the therapeutic options for patients with metastatic solid tumors, such as renal cancer. However, the development of relatively severe GVHD seems to be necessary to achieve tumor regression in the current setting. Thus, it is crucial to identify minor histocompatibility antigens (mHags) only expressed in tumor cells but not GVHD target organs. In this study, we examined whether three mHags: ACC-1 and ACC-2 encoded by *BCL2A1*, and HA-1 encoded by *HMHA1*, could serve as such targets for melanoma. Real-time PCR and immunohistochemical analysis revealed that the expression of both *BCL2A1*and *HMHA1* in melanoma cell lines and primary melanoma cells was comparable to that of hematopoietic cells. Indeed, melanoma cell lines were efficiently lysed by cytotoxic T lymphocytes specific for ACC-1, ACC-2, and HA-1. Our data suggest that targeting mHags encoded not only by *HMHA1*, whose aberrant expression in solid tumors has been reported, but also *BCL2A1* may bring about beneficial selective graft-versus-tumor effects in a population of melanoma patients for whom these mHags are applicable.

Keywords Minor histocompatibility antigen · Allogeneic hematopoietic stem cell transplantation · Melanoma

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1 Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) can cure hematopoietic malignancies. The success of donor leukocyte infusion or a non-myeloablative conditioning regimen demonstrated that the therapeutic effects of allogeneic HSCT mostly rely on the allogeneic immune responses. In an HLA-matched setting, allogeneic immune responses are mediated by donor-derived cytotoxic T lymphocytes (CTLs) against minor histocompatibility antigens (mHags). Ubiquitously expressed mHags are responsible for both life-threatening graft-versus-host disease (GVHD) and the graft-versus-leukemia (GVL) effect, whereas hematopoietic cell-restricted mHags, such as HA-1 [1] or ACC-6 [2], may be optimal target antigens which can potentially separate the GVL effect from GVHD development [3].

In some solid tumors, such as melanoma or renal cell carcinoma, there has been accumulating evidence that immunological manipulation, e.g., IL-2 [4, 5] or interferon treatment [6], can lead to clinical responses in some patients with refractory disease, although responses have been limited. Since the late 1990s, it has been reported that nonmyeloablative allogeneic HSCT leads to apparent tumor regression in these immunogenic solid tumors [7, 8]. However, the development of relatively severe GVHD seems to be necessary to achieve tumor regression in the