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*J Immunol* 2010; 185:7739-7745; Prepublished online 15 November 2010; doi: 10.4049/jimmunol.1001226
http://www.jimmunol.org/content/185/12/7739
Prediction of Reactivity to Noninherited Maternal Antigen in MHC-Mismatched, Minor Histocompatibility Antigen-Matched Stem Cell Transplantation in a Mouse Model

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The immunologic effects of developmental exposure to noninherited maternal Ags (NIMAs) are quite variable. Both tolerizing influence and inducing allorejection have been observed on clinical transplantation. The role of minor histocompatibility Ags (MiHAs) in NIMA effects is unknown. MiHA is either matched or mismatched in NIMA-mismatched transplantation because a donor of the transplantation is usually limited to a family member. To exclude the participation of MiHA in a NIMA effect for MHC (H-2) is clinically relevant because mismatched MiHA may induce severe alloreaction. The aim of this study is to understand the mechanism of NIMA effects in MHC-mismatched, MiHA-matched hematopoietic stem cell transplantation. Although all offsprings are exposed to the maternal Ags, the NIMA effect for the H-2 Ag was not evident. However, they exhibit two distinct reactivities, low and high responder, to NIMA in utero and during nursing depending on the degree of maternal microchimerism. Low responders survived longer with less graft-versus-host disease. These reactivities were correlated with Foxp3 expression of peripheral blood CD4+CD25+ cells after graft-versus-host disease induction and the number of IFN-γ-producing cells stimulated with NIMA pretransplantation. These observations are clinically relevant and suggest that it is possible to predict the immunological tolerance to NIMA. The Journal of Immunology, 2010, 185: 7739–7745.

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llogeneic hematopoietic stem cell transplantation (HSCT) is a curative treatment for various hematologic malignancies. Despite the presence of an increasing pool of unrelated volunteer donor registries, many patients who need allogeneic HSCT are not able to find a hematocompatible donor in proportion to the patients who have a rare HLA haplotype, but transplants from HLA-mismatched donors are limited by a number of historical barriers such as intractable graft-versus-host disease (GVHD) or graft failure (1).

There have been several previously reported investigations of noninherited maternal Ags (NIMAs) (2, 3). A reciprocal process, trafficking of maternal cells across the placenta, has also been documented (4, 5) and can result in life-long microchimerism in the offspring (6). In addition, maternal cells and HLA proteins are ingested by the baby during nursing, possibly stimulating oral tolerance (7, 8). However, the mechanisms by which NIMAs actually drive the immune system toward tolerance or rejection of allograft are still unclear. The clinical benefits of developmentally acquired tolerance to NIMAs were first noted by Owen et al. (9) 50 y ago. Since then, tolerogenic effects of NIMAs have been documented at both T and B cell levels in a variety of clinical settings (3, 10, 11).

In allogeneic stem cell transplantation, Van Rood et al. and others (12, 13) showed that the patients who received non-T cell-depleted (TCD) bone marrow transplantation (BMT) from a NIMA-mismatched donor had a significantly lower incidence of GVHD than noninherited paternal Ags. However, in non-TCD BMT from a NIMA-mismatched donor, 10% of patients still experienced severe acute GVHD (13). Furthermore, graft rejection and hyperacute GVHD in HSCT from NIMA-mismatched siblings were observed despite detecting of maternal microchimerism (MMc) (14). In contrast, Kanda et al. (15) described that a substantial proportion of long-term survivors after NIMA-mismatched HSCT could discontinue administration of immunosuppressive agents despite the frequent occurrence of moderate to severe chronic GVHD. Thus, the immunologic effects of developmental exposure to NIMAs are heterogeneous (8, 16, 17).

Although mismatch at minor histocompatibility Ags (MiHAs) can provoke severe immune responses against host cells upon transplantation (18), the role of MiHAs in NIMA effects has not been described. Recently, naturally acquired tolerance and sensitization to MiHAs have been reported (19). They showed the presence of MiHA-specific regulatory T cells in healthy adult women and men. In addition, it remains to be studied whether particular microchimeric cell types are associated with either a sensitized or a tolerized MiHA immunization status.

We generated H-2–mismatched and MiHA-matched NIMA-exposed model mice. Surprisingly, a tolerogenic NIMA effect was observed model mice. Surprisingly, a tolerogenic NIMA effect was
the degree of MMc. Moreover, we show the significant difference of IFN-γ-producing cells between NIMA-exposed and NIMA-nonexposed. These data indicate that it is possible to predict the tolerogenic effect of NIMA before HSCT.

Materials and Methods

Mice

C57BL/10 Sn Sk (B10, H-2d), B10.D2Sn Sn Sk (B10.D2, H-2d), and B10. BR/Sg Sn Sk (B10.BR, H-2b) mice were purchased from Japan SLIC (Shizuoka, Japan). Their background was all B10 type except H-2. To generate NIMA-exposed mouse models, we used the F2 breeding schema. A B10.BR male and (B10.D2xB10) F1 female were mated to generate H-2d offspring that were exposed to NIMA. H-2d offspring were exposed to NIMA-H2- in utero (Fig. 1A). NIMA-nonexposed mice were offspring of a B10.BR mother and a (B10.D2xB10) F1 father (Fig. 1B). In some experiments, we used another NIMA model generated with both H-2- and MiHA-mismatched combinations (Fig. 7A). They were nursed by the mother, weaned after 3 wk, and typed for the H-2 locus by flow cytometry using mAbs specific for H-2Kp and H-2Kd (BD Biosciences, San Diego, CA). These mice, aged 8–12 wk, were used for all experiments. The care and breeding of animals was in accordance with institutional guidelines.

Induction of GVHD

Sublethal GVHD was induced as described previously (20). Briefly, recipient B10 or B10.D2 female mice received 2 \( \times 10^8 \) spleen cells i.p. from NIMA-exposed and nonexposed mice after sublethal irradiation (550 cGy). The survival postinjection was monitored daily, and the degree of clinical GVHD was assessed by the scoring system that incorporates parameters: weight loss, posture, activity, fur texture, and skin integrity as described previously (21), grading from 0–2 for each parameter. A clinical index was subsequently generated by summation of the five criteria scores (maximum index = 10). It is known that this index is consistent with histopathologic findings for GVHD (21).

MLR-ELISPOT assay

We use peripheral blood (250–350 μl) for MLC with ELISPOT assay as described previously (26). Briefly, ELISPOT plates were coated with purified anti-IFN-γ, IL-4, and IL-10 mAb at 5 μg/ml and incubated overnight at 4°C. Plates were blocked for 2 h with RPMI 1640 medium containing 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml 2-ME, and 10% FBS. Responder cells (2 \( \times 10^5 \) /well) were incubated with the same number of 40 Gy-irradiated stimulator cells at 37°C with 5% CO2 for 72 h on the ELISPOT plate. After washing, biotinylated anti–IFN-γ, IL-4, and IL-10 mAb was added to 2 μg/ml (BD Biosciences) and incubated for 4 h at room temperature. After washing, streptavidin alkaline phosphatase (diluted 1/1000) was added for 2 h. After washing, spots were revealed by the addition of substrate solution. Visualized spot was counted by KS ELISPOT reader (Carl Zeiss, Tokyo, Japan).

Statistical analysis

Survival curves were plotted by using Kaplan-Meier estimates and compared by log-rank analysis. The Mann-Whitney U test was used for the
statistical analysis of clinical score, MMc, Foxp3, and ELISPOT data. A p value <0.05 was considered statistically significant. Data are presented as the means ± SE.

Results

No NIMA tolerogenic effect was evident

We examined the tolerogenic potential of NIMA exposure for H-2 of class I and II disparities without any influences of the MiHA (Fig. 1) (27, 28). In B10-recipient mice, a survival rate after receiving the transplant from B10.D2 allogeneic control donor mice was 28% at day 50, from NIMA-exposed donor mice (H-2<sup>d/k</sup>, NIMA<sup>a</sup>) was 34%, and from NIMA-nonexposed donor mice (H-2<sup>d/k</sup>) was 25% (Fig. 2A). In the B10.D2 recipient mice, a survival rate from B10 allogeneic control donor mice was 16% at day 50, from NIMA-exposed (H-2<sup>b/k</sup>, NIMA<sup>d</sup>) was 26%, and from NIMA-nonexposed (H-2<sup>b/k</sup>) was 11% (Fig. 2B). There are no significant differences among the groups, as shown in Fig. 2A and 2B. These data show that no NIMA-mediated tolerogenic effect was observed for the H-2 Ag. Contrary to previous reports that showed an apparent NIMA effect (2, 7), there was no evidence of a NIMA effect in the current results. The reason for the difference remains to be determined, but it could be due to the abrogation of the MiHA effect in our system.

Clearly different reactivities to NIMA in the current model

Accordingly, MLR was used to analyze whether the differences in the effects of NIMA underlie the individual responses to alloantigen. MLR, originally used to recognize a difference in the MHC class II, can be thought to detect an individual reactivity in NIMA-exposed mice because the current NIMA model developed from mice with mismatched MHC of both class I and class II. The proliferative response to allogeneic stimulation was higher in NIMA-exposed cells than in NIMA-nonexposed cells, and the NIMA-exposed mice were classified into high responders (HRs; more than mean +1 SD in NIMA-nonexposed) and low responders (LRs; less than mean +1 SD) (Fig. 3A, 3B). We used the mean +1 SD as the cutoff point due to the small sample size with a non-normal distribution. However, when the mean +2 SD was used as the cutoff, it did not affect the conclusion. In B10-recipient mice, survival rates of NIMA-exposed LR mice (H-2<sup>d/k</sup>, NIMA<sup>a</sup>) were significantly better than those of NIMA-exposed HR mice (53 versus 18%; p = 0.001) (Fig. 3C). Similarly, in B10.D2 recipients, the survival in those receiving transplants from NIMA-exposed LR mice (H-2<sup>b/k</sup>, NIMA<sup>d</sup>) was significantly better than those from NIMA-exposed HR mice (38 versus 8%; p = 0.028) (Fig. 3D). Thus, the MLR reactivity was in parallel with the strength of

FIGURE 3. Two distinct reactivities to H-2 in NIMA-exposed mice resulted in differences in GVHD induction. The mice were classified into two groups based on their reactivity to NIMA; the HR (≥ mean +1 SD in NIMA-nonexposed) or the LR (< mean +1 SD) group. A. The PBMCs from NIMA-exposed H-2<sup>d/k</sup> mice (n = 34) and NIMA-nonexposed H-2<sup>d/k</sup> mice (n = 19) as responders were stimulated with B10 (allogeneic) and B10.D2 (semisyngeneic) mouse PBMCs. B. NIMA-exposed H-2<sup>b/k</sup> mice (n = 26) and NIMA-nonexposed H-2<sup>b/k</sup> mice (n = 11) as responders were stimulated with B10 (semisyngeneic) and B10.D2 (allogeneic) mouse PBMCs. C. The survival of sublethally irradiated recipient B10 female mice injected with cells from NIMA-exposed LR mice (H-2<sup>d/k</sup>, NIMA<sup>a</sup>) (○, n = 30), HR mice (NIMA<sup>a</sup>) (△, n = 33), and NIMA-nonexposed mice (●, n = 39). D. The survival of sublethally irradiated recipient B10.D2 mice injected with cells from NIMA-exposed LR mice (H-2<sup>b/k</sup>, NIMA<sup>d</sup>) (○, n = 18), HR mice (NIMA<sup>d</sup>) (△, n = 12), and NIMA-nonexposed mice (H-2<sup>b/k</sup>) (●, n = 9). E. Body weights were determined in recipient injected with cells from NIMA-exposed LR donor mice (○, n = 48), NIMA-exposed HR donor mice (△, n = 45), allogeneic donor mice (●, n = 47), and vehicle (Ο, n = 12). F. The clinical GVHD score was determined in recipients injected with cells from NIMA-exposed LR mice (○, n = 48), NIMA-exposed HR mice (△, n = 45), allogeneic mice (●, n = 47), and vehicle (Ο, n = 12). Data are expressed as the means ± SE of individual animals. *p < 0.05; **p < 0.01.
the NIMA effect. It was suggested that the survival rates were dependent on the severity of GVHD. The body weight loss of the recipients from NIMA-exposed LR mice was significantly less severe than those from NIMA-exposed HR mice at 10 and 14 d after GVHD induction (Fig. 3E). The clinical score from the NIMA-exposed LR mice was significantly lower than those from NIMA-exposed HR mice at different time points (Fig. 3F). Collectively, these results clearly showed that NIMA-exposed mice could be classified into two groups (HRs and LRs) by their differences in GVHD induction.

**Correlation between MMc and the tolerogenic NIMA effect**

MMc occurs in at least half of all humans from fetal life into adulthood (6, 29). The factors that govern MMc are poorly understood, but may be of considerable clinical relevance in view of the fact that MMc has been associated with autoimmunity and allograft tolerance (30). We analyzed the levels of MMc in young adults of NIMA-exposed mice using nested PCR for MHC class II Eβ-specific DNA. The NIMA-exposed LR and HR mice had positive signals of varying intensity when compared with a titration mixture of B10 and B10.D2 peripheral blood cells at concentrations ranging from 1/1 to 1/10⁵ (Fig. 4A). MMc was recognized in 15 out of 15 of the NIMA-exposed LRs and 8 out of 10 of the NIMA-exposed HRs (Fig. 4B). The level of MMc in the LR group was significantly higher (p < 0.001) than that in the HR group, when the quantitative MMc was calculated using an image analyzer (Fig. 4C). These results suggest that the level of MMc was correlated with tolerogenic effect of the NIMA, supporting the recent report (30).

**Fopc3 expression and regulatory cell after GVHD induction**

Using real-time PCR, we found that the levels of Foxp3 RNA expression isolated from the recipient mice of NIMA-exposed LR (n = 5) transplants to be significantly higher than those in the recipients from NIMA-exposed HR (n = 5) and NIMA-nonexposed (n = 5) donors. When the recipient mice were analyzed by flow cytometry, the number of Foxp3⁺CD4⁺CD25⁺ cells increased in the NIMA-exposed LRs. The origin of Foxp3 was limited in CD4⁺ cells, but not in CD8⁺ cells, B cells, NK cells, and monocytes/macrophages (Fig. 5B). These results were in agreement with the fact that MMc is closely associated with Foxp3 expression at a point of tolerance induction, supporting the recent report (30). Taken together, the NIMA tolerogenic effect was associated with Foxp3 expression and the induction of regulatory cells in a NIMA mouse model with an H-2-mismatched and MiHA-matched combination.

**Prediction of a tolerogenic NIMA effect by MLR-ELISPOT assay**

To further examine the immunological mechanism underlying the tolerogenic NIMA effect in vitro, we examined the frequency of cytokine-producing alloreactive cells using an ELISPOT assay combining with MLR. We detected a potent response by IFN-γ-producing cells from the NIMA-exposed LRs, HRs (H-2<sup>kk</sup>, NIMA<sup>B</sup>), and NIMA-nonexposed mice (H-2<sup>kk</sup>) to B10 stimulator cells. The response to the NIMA-exposed LR mice showed a lower IFN-γ production compared with the NIMA-exposed HR mice (40.2 spots versus 70.5 spots; p < 0.01) and NIMA-nonexposed mice (versus 80.1 spots; p < 0.01) (Fig. 6). We also examined the frequency of IL-4- and IL-10-producing alloreactive cells that were not detectable (data not shown). Moreover, we described the predictive reactivity in another NIMA model with major- and minor-mismatched combination (Fig. 7A). These mice were affected by the effects of both major and minor NIMA as the conventional NIMA model described previously (2, 31). The response to NIMA-exposed mice (H-2<sup>kk</sup>, NIMA-H<sup>B</sup>–H<sup>M</sup>A<sup>S</sup>, n = 10) showed lower IFN-γ production compared with NIMA-nonexposed mice (H-2<sup>kk</sup>, n = 7) (33.9 spots versus 96.9 spots; p < 0.01) and allogeneic mice (versus 128.0 spots; p < 0.01) (Fig. 7B). Collectively, these results demonstrate that it is possible to predict the immunological tolerance to NIMA in major- and minor-mismatched transplants.

**Discussion**

Several reports described the tolerogenic effect to NIMA in mouse model that is in both H-2- and MiHA-mismatched settings (2, 7, 31). The association of tolerance to NIMA with MMc also was confirmed (2, 30). However, the immunologic effects of developmental exposure to NIMA are quite variable by different settings (16). The precise mechanisms of the heterogeneity are still under investigation. The relevance of MiHAs in NIMA effect has not been reported. Not only in the MHC-identical but also in the MHC-haploidentical situation, MiHA alloreactivities may be induced upon transplantation (32). Therefore, the focus on the
NIMA effect to H-2 is clinically relevant. We assessed NIMA effect in MHC-mismatched, MiHA-matched HSCT in the current study. Besides, the results may be useful for predicting reactivity to a NIMA-mismatched donor in the clinical setting.

Our study clearly showed the difference of individual reactivities toward H-2, not MiHA. The difference of reactivity to NIMA was critically influenced by the amount of MMc expression. The relationship between tolerogenic effect and MMc is consistent with a report of Dutta et al. (30). They described the correlation between MMc and NIMA-specific regulatory T cells capable of suppressing both delayed-type hypersensitivity and lymphoproliferative responses of effector T cells in conventional NIMA mouse model. Opiela et al. (33) described that transient exposure to low levels of NIMA alloantigens in early life may lead to long-term priming for both cytotoxic and Th cell functions. In contrast, Aoyama et al. (7) showed that both oral and in utero exposures to NIMA are required for the maximum induction of tolerance. In any case, the mechanism underlying the development of tolerance versus priming to NIMA alloantigens remains to be investigated.

We demonstrated, for the first time to our knowledge, the individual difference of NIMA tolerogenic effect, HR or LR, associated with Foxp3 expression by using an original NIMA mouse model with H-2–mismatched, MiHA-matched. Although Foxp3 is not a very specific marker for regulatory T cells given that activated nonregulatory T cells may transiently upregulate this transcription factor (34, 35), several reports and current data described that regulatory T cells mediate tolerance to NIMA (31, 36). whereas in the previous NIMA model, F1 backcross breeding model (B6xBDF1), H-2b/b-type offspring to NIMA-d as well as MiHA-MiHA is a part of DBA/2 mice background (2, 31), offspring in our model was all B10 mice background except H-2 by using B10 congenic mice. Previous reports proved a tolerogenic effect for NIMA in the former conventional model (2, 31). However, the tolerogenic effect has not been detected in the current model by excluding the involvement of MiHA.
The mouse MiHA loci conferred a wide range of immunogenicity ranging from weakly to strongly immunogenic (37). More than 50 murine MiHA loci have been identified with theoretical estimates of several hundred (38). However, there is not really much MiHA causing or contributing to GVHD. Recent studies have provided evidence that GVHD could be caused by only a limited number of MiHA, including H13, H4, H7, H28, H60, and H-Y (39–41). This MiHA immunodominance was manifested on genetically varied backgrounds among B10, BALB/c, and DBA/2 strains (27, 42, 43). In the current study, all B10 congenic mice were used as NIMA models, and those MiHAs had matched entirely in this system. Therefore, a current NIMA mouse model, but a conventional NIMA model, did not affect immunogenicity of MiHA. It might be difficult to get the individual difference of NIMA tolerogenic effect in MiHA, but not H-2.

Frequencies of CTL precursor (CTLp), Th lymphocyte precursor (HTLp), and MLR were reported as methods to detect an individual reactivity to NIMA in vitro to date (44–46). Besides the analysis of CTLp and HTLp frequencies and MLR, which are also contradictory, an established test system has not been available for the prediction of outcome after HSCT. Originally, CTLp reflects alloreactivity of class I mismatch, and MLR and HTLp reflect alloreactivity of class II mismatch. In the current study, we used MLR assay for evaluating the alloreactivity of NIMA-exposed mice, which showed quite a wider range of reactivity than that of NIMA-nonexposed. It means that fetomaternal interaction acts on both tolerance (LR) and sensitization (HR) certainly (16, 19). The reports from Tsafir et al. (46) and Falkenberg et al. (44) were detecting a reactivity to NIMA by MLR and CTLp and HTLp, respectively. Interestingly, although we scrutinized their figures, individual reactivities of NIMA group showed a wider range than control group, and those reactivities seem to be divided into low and high reaction, although they did not mention it. In this way, reactivity to NIMA could be detected in vitro, and either tolerance or sensitization could be determined by a condition of fetomaternal interaction. A reciprocal trafficking of maternal and fetal cells across the placenta (4) can lead to persistent MMc associated with an Ag-specific suppression of the T cell responses (12). Presence of fetomaternal tolerance was expected by expression of MMc (2), although they did not mention it. In this way, reactivity to NIMA could be detected in vitro, and either tolerance or sensitization could be determined by a condition of fetomaternal interaction. A reciprocal trafficking of maternal and fetal cells across the placenta (4) can lead to persistent MMc associated with an Ag-specific suppression of the T cell responses (12). Presence of fetomaternal tolerance was expected by expression of MMc (2). In addition, NIMA alloantigens are thought to induce tolerance and protection from allergic asthma. N. Engl. J. Med. 339: 1657–1664.

Disclosures
The authors have no financial conflicts of interest.

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