Chloroquine Treatment Affects T-Cell Priming to Minor Histocompatibility Antigens and Graft-Versus-Host Disease

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Graft-versus-host disease (GVHD) caused by T-cell recognition of minor histocompatibility (MiHC) antigens is a major complication of bone marrow transplantation (BMT). Although a cytokine cascade induced by the preparative regimen leading to secondary T-cell activation as the cause of GVHD has been hypothesized, there is strong evidence that GVHD is a result of donor T-cell recognition of minor histocompatibility (MiHC) antigens expressed by recipient cells when both donor and recipient are major histocompatibility complex (MHC)-identical. Donor T-cell responses require MHC-identical recipients to express one or more minor histocompatibility gene product that is not identical and results in a clonal proliferation of T cells in vivo.

In mice, transplantation of T-cell subpopulations from donor mice into MiHC-different recipients have shown that both CD4+ and CD8+ T-cell subpopulations are required to develop GVHD. In humans, donor-derived CD4+ T-cell clones directed to recipient MiHC and isolated from BMT patients with or without acute and chronic GVHD correlate with the development of GVHD. Antihost cytotoxic CD8+ T cells have also been observed in the first 3 to 6 months after BMT in patients with GVHD, but with poor correlation. Thus, both CD4+ and CD8+ T-cell populations reactive with MiHC appear to be important for development of GVHD in both mice and humans.

CD4+ T-cell activation is dependent on antigen presentation in the context of class II MHC. costimulatory molecules such as B7/CTLA-4 expressed on antigen-presenting cells, and cytokines produced by accessory cells. Presentation by MHC class II-expressing cells is usually through an exogenous pathway whereby peptide, after endocytosis, binds intralysosomally with class II MHC after the MHC molecule has dissociated from the invariant chain, a chaperone protein for both α and β chains of class II MHC. Endogenously produced MiHC peptides can also bind to class II MHC by the endogenous pathway. At this time, there is evidence that the MHC class II-expressing antigen-presenting cells from the donor’s transplanted immune system are important for the development of GVHD and tolerance induction, and recently it has been shown that blockade of MHC class II in vivo using a high-affinity peptide can inhibit GVHD in mice. In addition, we have recently shown that the absence of B cells, a major MHC class II antigen-presenting cell population, in vivo results in decreased T-cell priming to MiHC and to development of GVHD secondary to MiHC differences. Non-specific depletion or suppression of donor T cells has represented the most prevalent approach to control GVHD.

Modulation of MHC class II antigen presentation in vivo may represent an alternative approach to decrease T-cell responses to MiHC and affect the development of GVHD.

Chloroquine is known to block MHC class II-dependent antigen processing and presentation. In vitro, it appears to block processing by affecting lysosomal acidification, resulting in decreased invariant chain dissociation from the MHC class II molecule and a lack of antigen binding to class II MHC. Chloroquine treatment in vivo is an established immunosuppressive therapy of autoimmune diseases such as juvenile rheumatoid arthritis and systemic lupus erythematosus, although the mechanism by which it acts is unclear. Other investigations have shown that chloroquine treatment of mice can result in a decrease in MHC class II antigen expression in vivo and can result in decreased tumor...
necrosis factor levels. Thus, chloroquine clearly is able to block MHC class II processing and presentation in vitro and has immunosuppressive effects in vivo presumably by the same mechanisms. We hypothesized that chloroquine can be used to explore whether inhibition of class II MHC presentation of MiHC in vivo has an effect on T-cell activation to MiHC and the development of GVHD.

We evaluated whether chloroquine administered in vivo can inhibit T-cell activation to MiHC and whether this mechanism is through the inhibition of MHC class II presentation of MiHC. In addition, we evaluated whether inhibition of MHC class II presentation of MiHC has an effect on the induction of GVHD. We found that chloroquine inhibits the onset of GVHD apparently by decreasing CD4+ and CD8+ T-cell activation to MiHC.

MATERIALS AND METHODS

Mice and materials. Four- to six-week-old female C57BL/6 (H-2b) mice, LP/J (H-2d) mice, C3H.SW (H-2b) mice, and BALB/c (H-2k) were obtained from Jackson Laboratories (Bar Harbor, ME) and were maintained in the animal care facility at the Children’s Variety Research Center. BALB.B (H-2d) mice were obtained as a kind gift of Dr P.D. Greenberg (University of Washington, Seattle, WA).

Reagents. Chloroquine, lipopolysaccharide (LPS), and concanavalin A (con A) were purchased from Sigma Chemical Co (St Louis, MO). A fresh preparation of chloroquine was suspended in sterile phosphate-buffered saline (PBS) before each experiment at a final concentration of 2 mg/mL (400 μg/200 μL) for intraperitoneal (IP) injection. Anti-MHC class II-2’-fluorescein isothiocyanate (FITC) and antimouse IA-FITC monoclonal antibodies (MoAbs) were purchased from Pharmingen (San Diego, CA). Antimouse Thy 1.2-FITC, Lyt-2–FITC, and L3T4-phycoerythrin MoAbs were purchased from Becton Dickinson (San Jose, CA). Glutaraldehyde was purchased from J.T. Baker Chemical CO (Phillipsburg, NJ).

Immunophenotyping. Cell labeling was performed on 1 × 10^6 cells in a 100-μL volume of Matix Medium defined as 1:1 ratio of RPMI-enriched eagle’s medium and was supplemented with 10% fetal bovine serum, 1 × 10^-4 mol/L 2-mercaptopethanol, 10 mmol/L L-glutamine, 20 μg/mL gentamycin, and 10 mmol/L HEPES buffer at 4°C for 30 minutes with the indicated antibodies. After fluorescent labeling, cells were washed 3 times with 1 mL of Matix media and fixed in 2% paraformaldehyde before analysis. Immunofluorescent analysis was performed on a Coulter Profile II fluorescent cell sorter (Coulter Inc, Hialeah, FL).

T-cell priming for minor histocompatibility antigens, proliferation, and cytotoxicity. C57BL/6 mice were primed by IP inoculation with 2.5 × 10^5 viable red blood cell-lysed BALB.B spleen cells. Spleens were collected 4 to 8 weeks later and were evaluated for proliferative activity. LP/J and C3H.SW mice were also similarly primed with 2.5 × 10^5 C57BL/6 spleen cells, followed by removal of spleen cells 4 to 8 weeks later for evaluation of proliferation in vitro.

For the proliferative assay, spleen cells were obtained from mice previously primed as described above. Responder cells (5 × 10^5 cells/well) were cultured in a 196-well U-bottom plate in Matix media with irradiated (2,500 cGy) stimulator cells at responder-to-stimulator (R:S) ratios of 2:1, 4:1, and 8:1. The stimulator cells included an allogeneic-positive control (ie, BALB/c spleen cells), the MiHC-incompatible cells that were administered in the previous immunization (ie, BALB.B), and a syngeneic-negative control. Responder cells (in triplicate) were cultured in vitro for 4 days with irradiated stimulators, were pulsed with 1.0 μCi [3H]thymidine (Amersham, Oakville, Ontario, Canada) on day 4 and were harvested using a PHD Cell Harvester (Cambridge Technologies, Watertown, MA) 18 hours later, and were counted in a liquid scintillation counter. Data are presented as the mean difference, with standard error of the mean (SEM) bars in stimulated and unstimulated cultures (Δ CPM).

Cytotoxicity was evaluated in a chromium-release assay performed after 4 days of in vitro restimulation. Responder cells from C57BL/6 mice primed to BALB.B cells in vivo (6 × 10^6 cells/well) were cultured with 1.25 × 10^6 cells/well (R:S = 4:1) of irradiated BALB.B stimulators (2,500 cGy) in 24-well plates (Costar, Cambridge, MA) for 4 days and were removed for evaluation in a cytotoxicity assay. Cytotoxicity of the effector cells was evaluated in a standard 4-hour 51Cr-release assay with labeled con A-stimulated blast targets. Effector-to-target ratios were at 80:1, 20:1, and 5:1. The percent specific lysis was evaluated by measuring the experimental minus spontaneous 51Cr released. Specificity was shown by the ability of the effectors to lyse the target previously immunized for (BALB.B) and not another MHC-identical nonspecific con A blast from a C57BL/6 mouse.

Purification of T-cell populations used in proliferative assays. Responder T cells were obtained from red blood cell-lysed spleen cells passed over a goat antimouse IgG (H+L) glass bead column (Cellect, Biotex Laboratories, Edmonton, Alberta, Canada). Eluted cells were collected in a 5-mL volume at a flow rate of 7 drops per minute. The purity of T cells was determined by phenotyping with anti-Thy 1.2 FITC-conjugated antibody as well as in a mitogen assay using con A and LPS.

Graft-versus-host model. Recipient C57BL/6 mice were treated with chloroquine at 400 μg (200 μL) or PBS at 200 μL IP for 5 days before transplantation and then were irradiated with 950 cGy (total body) from a 137Cesium source 12 to 24 hours after the last dose. We used a previously described and well characterized GVHD model dependent on both CD4+ and CD8+ T cells for development of GVHD. A total of 107 Thy-1-depleted bone marrow cells (anti-Thy 1.2 MoAb + complement depletion as previously described) and approximately 5 × 10^6 whole population spleen cells with 2 × 10^5 Thy 1+ cells from LP/J mice were infused intravenously into each of the irradiated C57BL/6 recipients. Chloroquine at 400 μg (200 μL) or PBS at 200 μL IP was administered once or twice weekly until the end of the experiment. To ensure no GVHD-like toxicity from prolonged chloroquine infusion, one group of mice received an infusion of C57BL/6 donor cells (syngeneic) followed by treatment with chloroquine at 400 μg IP twice per week. Mice were maintained in a low microbial environment microisolator cages for 21 days after transplant and then were exposed to a nonsterile environment after that time. With each experiment, a group of recipient mice received irradiation alone as a control for graft failure. All of these mice died by 20 days post-BMT. Evaluation of GVHD was based on the clinical findings of hunched appearance, ruffled hair, wasting, wrinkled ears, and hair loss followed by death. Mice identified as having GVHD were killed within 72 hours of projected death to minimize suffering as per criteria established by the University of British Columbia Ethical Review Committee.

Serum interleukin-2 (IL-2) levels. Serum IL-2 levels were evaluated using the InterTest-2X Mouse ELISA Kit obtained from Genzyme (Cambridge, MA). Briefly, diluted or undiluted serum was aliquoted in duplicates into microtiter test wells that were precoated with antimouse IL-2. After a 40-minute incubation at 37°C, the wells were washed 4 times, incubated with biotinylated polyclonal antimouse IL-2 antibody for 40 minutes at 37°C, rewash, and further incubated with Streptavidin Peroxidase for 25 minutes at 37°C. The wells were then washed and incubated with the substrate, tetramethylbenzidine for 10 minutes. The reaction was stopped with 1 mol/L sulfuric acid, and the plates were immediately read on a BioTek ELISA plate reader (Bio-Tek Instruments, Inc, Winooski, VT) at 450 nm.
Glutaraldehyde fixation. Cells were fixed with glutaraldehyde as previously described. C57BL/6 spleen stimulator cells were suspended at 5 x 10^6 cells/mL in PBS and added to PBS containing 0.1% glutaraldehyde at a 1:1 mixture. After 30 seconds, cells were washed twice with 100:1 vol of PBS and centrifuged before resuspension in Matris media for use in a proliferation assay.

Statistical methods. Mean values and SEM of the counts per minute were calculated using routine methods. Statistical significance was calculated using the unpaired Student’s t-test on mean values and the Fisher’s exact two-tailed test for discrete variables.

RESULTS

Effect of chloroquine treatment on T-cell proliferative responses to minor histocompatibility antigens. Initially, we evaluated the effect of different chloroquine treatment regimens on T-cell priming to MiHC using a well-characterized MhC-incompatible model (C57BL/6 anti-BALB.B). This response is dependent on CD4^+ T cells for both a proliferative and cytolytic response. After pretreatment with chloroquine at 400 μg (200 μL) IP weekly for 2 or 3 weeks or daily for 5 days, C57BL/6 (H-2^b) mice were primed IP with 2.5 x 10^7 viable BALB.B (H-2^b, MiHC-incompatible) spleen cells. Control mice received 200 μL of PBS IP daily for 5 days. Mice were primed within 24 hours of the last chloroquine dose. After inoculation of BALB.B cells, all mice were treated with weekly doses of chloroquine at 400 μg or with 200 μL of PBS IP until the end of the experiment. Proliferation of spleen cells from C57BL/6 mice primed with BALB.B cells (Fig 1A) was reduced by 41% in those pretreated weekly with chloroquine for 2 weeks or 3 weeks as compared with that of those receiving PBS (P = .03). An even greater decrease of 67% was observed in mice pretreated daily for 5 days (5/wk; P = .008).

We also evaluated the effect of chloroquine on T-cell priming to MiHC using a second minor histocompatibility-incompatible response (LP/J anti-C57BL/6). This model represents a well-established graft-versus-host model (LP/J donor into C57BL/6 recipient). Because pretreatment with chloroquine for 5 days before priming with spleen cells had the greatest effect in the first experiments, we used that schedule followed by 400 μg chloroquine either once (1/wk) or twice (2/wk) per week after priming. LP/J (H-2^d) mice were primed with 2.5 x 10^7 viable C57BL/6 (H-2^d, MiHC-incompatible) spleen cells and evaluated 4 to 8 weeks later (Fig 1B). Spleen cells from primed LP/J mice treated with chloroquine at 400 μg twice per week (2/wk) after priming again showed a significant decrease in proliferation of 57% as compared with that of PBS-treated controls (P = .04). Thus, chloroquine significantly decreased the proliferative T-cell response in two separate minor histocompatibility antigen-incompatible models.

We then evaluated the relative importance of chloroquine treatment before priming compared with that after priming using the C57BL/6 anti-BALB.B response (Fig 1C). We primed mice exactly as in Fig 1A. Mice received chloroquine at 400 μg IP daily for 5 days before priming followed by 400 μg IP weekly for 4 weeks as in the experiments in Fig 1A. In addition, mice received chloroquine as 400 μg IP daily for 5 days before priming followed by no chloroquine after priming or by chloroquine at 400 μg IP weekly for 4 weeks with none administered before priming. Significant proliferation was seen only in the PBS-treated group, with very little response observed in any of the other three experimental groups. Therefore, there appeared to be more suppression when mice were treated with chloroquine before priming as compared with that with chloroquine treatment only after priming, although this difference was not significant. Thus, chloroquine treatment after priming is able to suppress the C57BL/6 anti-BALB.B response. All subsequent experiments presented used both chloroquine treatment before priming (or BMT) followed by chloroquine treatment until the end of the experiment to ensure a consistent effect by chloroquine.

Effect of chloroquine treatment on T-cell cytolytic responses to minor histocompatibility antigens. To evaluate the effect of chloroquine on a cytolytic T-cell response, we used the well-characterized C57BL/6 anti-BALB.B cytolytic response. Mice received 400 μg of chloroquine or PBS daily for 5 days before priming with BALB.B cells and were then administered chloroquine at 400 μg once per week, 400 μg 3 times per week, or PBS three times per week (Fig 2). Specific lysis in both chloroquine-treated groups was decreased by greater than 50% and was significantly lower (P < .05) as compared with that of PBS-treated mice except at the highest effector-to-target (80:1) ratio in the lower chloroquine dosing schedule. Background lysis of a C57BL/6 con A target is shown as the control target (Fig 2). Thus, chloroquine treatment induced a dose-dependent decrease in cytolytic T-cell activity against MiHC.

Effect of chloroquine therapy on T- and B-cell function. Chloroquine-induced inhibition of T-cell activation to MiHC antigens in vivo presumably is secondary to decreased MHC class II presentation of MiHC. It is possible that chloroquine has a direct nonspecific effect on T cells resulting in decreased T-cell responses in general. To exclude this possibility, we evaluated nonspecific T- and B-cell responses to mitogen and T-cell allogeneic responses. These evaluations were performed at the time of priming and 4 weeks later at a time similar to when the proliferative and cytolytic assays were performed in the previous experiments (Figs 1 and 2). Spleen cells from C57BL/6 mice treated with 400 μg of chloroquine weekly for 2 or 3 weeks or daily for 5 days were evaluated for function at the same time as priming was performed previously (Fig 1A). All groups had normal proliferative responses to an allogeneic stimulator (BALB/c; H-2^d; see Fig 3A). Similarly, LPS-induced proliferation did not differ in all groups (Fig 3A), showing that chloroquine treatment had no effect on B-cell responses. Interestingly, chloroquine-treated mice had an increased proliferative response to a T-cell mitogen, con A, after 5 days of chloroquine treatment (5/wk). The frequency of CD3-, CD4-, CD8-, Thy 1-, and IgM-bearing cell studies by immunofluorescence analysis were the same in control and chloroquine-treated mice (data not shown). Thus, chloroquine treatment did not induce nonspecific suppression of T cells at the time of priming.

We repeated these evaluations 4 weeks later after chloroquine treatment in a manner identical to that used previously in the proliferative and cytolytic assays (Figs 1 and
Fig 1. Effect of chloroquine treatment in vivo on T-cell proliferative responses to MiHC. (A) C57BL/6 mice were treated with chloroquine at 400 μg (200 μL) IP weekly for 2 weeks (2 wk), weekly for 3 weeks (3 wk), or daily for 5 days (5/wk), with priming 12 to 24 hours after the last chloroquine dose. Control mice received PBS at 200 μL IP daily for 5 days (PBS). C57BL/6 mice were primed by IP injection with 2.5 x 10⁷ viable spleen cells from BALB.B mice (H-2b, MiHC-incompatible). Mice were treated with 400 μg chloroquine IP weekly after priming and were evaluated more than 4 weeks after priming using [³H]-thymidine incorporation assay with irradiated stimulators. Results are shown as the proliferation minus the background (Δ CPM). This figure is a representative experiment of three separate experiments. (B) LP/J mice were primed with spleen cells from C57BL/6 (H-2b, MiHC-incompatible) identically to that in (A). Mice were treated with chloroquine at 400 μg IP daily for 5 days followed by 400 μg IP once per week (1/wk) or twice per week (2/wk) or with PBS at 200 μg twice per week (PBS). Priming response was evaluated 4 to 8 weeks after priming as in (A). This figure is representative of two experiments. (C) C57BL/6 mice were treated with chloroquine at 400 μg (200 μL) IP daily for 5 days before priming, followed by once weekly injections for 4 weeks; daily injections for 5 days before priming, with no injections after priming; or once weekly injections for 4 weeks after priming, with no injections before priming. Controls received PBS at 200 μL IP daily for 5 days, followed by weekly injections for 4 weeks afterwards. Mice were primed exactly as in (A). This figure represents one experiment.

2). This way we could exclude any effect of prolonged chloroquine treatment on T-cell function. C57BL/6 mice were treated with chloroquine for 5 days followed by chloroquine treatment either once or twice weekly for 4 weeks (Fig 3B). No effect of chloroquine treatment on proliferation to an allogeneic stimulator (BALB/c) or to the B-cell mitogen, LPS, (Fig 3B) was observed. Unlike the con A mitogen response observed at the time of priming (Fig 3A), we saw no effect of chloroquine on the T-cell mitogen response after chronic administration of chloroquine (Fig 3B). Thus, chloroquine treatment had no nonspecific suppression of T-cell function after either short or prolonged administration, and it is unlikely that this is the reason for the decreased T-cell responses to MiHC observed in Figs 1 and 2.

Effect of chloroquine treatment on the development of GVHD. To evaluate whether chloroquine treatment affected GVHD secondary to T-cell recognition of MiHC, we selected the murine GVHD model of an LP/J donor into C57BL/6 recipient (H-2b, MiHC-incompatible), which is dependent on both CD4⁺ and CD8⁺ T cells. C57BL/6 recipient mice were treated daily with 400 μg of chloroquine or with 200 μL of PBS (control) for 5 days before transplantation. Posttransplantation treatment consisted of chloroquine at 400 μg IP once or twice per week or PBS at 200 μL twice per week until the end of the experiment. A control group consisted of mice receiving chloroquine at 400 μg IP daily for 5 days before BMT followed by 400 μg twice weekly for those that received syngenic donor cells (C57BL/6). This group ensured that prolonged chloroquine treatment did not induce symptoms similar to those of GVHD. These mice were healthy at the end of the experiment. All mice that were irradiated (950 cGy) with no donor cells infused died by day 20 after transplantation. All of the mice that died (Fig 4) had clinical evidence of GVHD (hair loss, hunched appearance, and wrinkled ears) excluding the irradiation controls, and all of the mice that survived appeared well at the end of the experiment.

GVHD developed in 2 of 12 (17%) mice that received
chloroquine twice per week after transplantation and in 1 of 6 (17%) of those treated with chloroquine once per week after transplant (Fig 4), which was significantly less frequent than that in PBS-treated controls (12 of 13 [92%]; P < .001). Thus, chloroquine treatment significantly decreased the frequency of GVHD after BMT secondary to MiHC differences.

Effect of chloroquine treatment on MHC class II surface expression and IL-2 serum levels. Having established that chloroquine treatment decreases both proliferative and cytolytic T-cell responses to MiHC and decreases the incidence of GVHD without any direct effect on T- or B-cell function, we wished to evaluate other possible mechanisms that chloroquine may have in vivo. Previously, it was shown that chloroquine treatment in mice was because of decreased MHC class II antigen presentation after chloroquine treatment in mice was because of decreased MHC class II cell surface expression in vivo. We evaluated MHC class I and II expression by spleen cells after treatment with PBS or chloroquine at 200 μg or 400 μg IP daily for 5 days. Spleen cells were removed between 12 to 24 hours after the last IP dose and were evaluated by immunophenotyping. We looked at both the percentage of cells positive for either marker and the mean intensity on the positive cells. Short term chloroquine treatment (daily for 5 days) had no effect on MHC class I (H-2D<sup>b</sup>) or MHC class II (I<sup>a</sup>) expression as compared with that of PBS-treated controls (Table 1). Similarly, prolonged chloroquine administration at 400 μg IP either once or three times weekly for 4 weeks resulted in no effect on MHC class II or I expression.

Serum IL-2 levels may also be decreased after chloroquine

![Graph](https://via.placeholder.com/150)

Fig 2. Effect of chloroquine treatment in vivo on T-cell cytolytic responses to MiHC. C57BL/6 mice were primed with BALB.B cells as in Fig 1A and were treated with chloroquine at 400 μg or PBS at 200 μL IP daily for 5 days, followed by priming within 12 to 24 hours. Chloroquine was administered as 400 μg IP weekly (low-dose group) or three times per week on Monday, Wednesday, and Friday (high-dose group), or PBS was administered as 200 μL 3 times per week (PBS). Spleen cells were removed and evaluated in a chromium-release assay against BALB.B con A blasts after 4 days of restimulation in vitro with irradiated BALB.B stimulators.

![Graph](https://via.placeholder.com/150)

Fig 3. Effect of chloroquine treatment in mitogen and allogeneic responses. (A) C57BL/6 mice were treated with chloroquine at 400 μg IP weekly for 2 weeks (2 wks), weekly for 3 weeks (3 wk), or daily for 5 days (5/wk) or with PBS at 200 μL daily for 5 days (PBS) as in Fig 1A and were evaluated for function at the time of priming. T-cell response was evaluated by incubation of 5 × 10<sup>5</sup> C57BL/6 spleen cells with irradiated allogeneic stimulator BALB/c cells (H-2<sup>b</sup>) at an R:S ratio of 1:1 and were evaluated for proliferation by [3H]-thymidine incorporation in the last 18 hours after 4 days. T-cell proliferation to con A at 2.5 μg/mL and B-cell responses to LPS at 5 μg/mL were evaluated by incubation of 2.5 × 10<sup>5</sup> cells/well in a 96-well U-bottomed plate and were evaluated for [3H]-thymidine incorporation as before in a 4-day culture. This figure is representative of three experiments. (B) Mice were treated exactly as above except that treatment was continued for an additional 5 weeks as in the previous priming experiments. C57BL/6 mice were treated with chloroquine at 400 μg IP daily for 5 days in the first week, followed by weekly injections once weekly (1/wk) or twice weekly (2/wk). PBS was administered at 200 μL in the same schedule as the mice in the 2/wk chloroquine group. Responses to allogeneic stimulators and mitogens were performed exactly as in Fig 3A. This figure is representative of two experiments.
CHLOROQUINE AND GVHD

Effect of chloroquine treatment on the development of GVHD after BMT (LP/J donor into C57BL/6 recipient). C57BL/6 recipient mice treated with chloroquine at 400 μg (or 200 μL PBS) IP for 5 days were irradiated (850 cGy), followed by infusion of 1 × 10⁸ LP/J bone marrow cells and additional spleen cells containing 2 × 10⁷ Thy 1 T cells. After transplantation, mice were injected with chloroquine at 400 μg IP once weekly or twice weekly, or PBS at 200 μL was administered twice weekly until the end of the experiment. Controls included C57BL/6-recipient mice transplanted with C57BL/6 donor cells and treated with chloroquine at 400 μg as described above (syngeneic controls) and mice which received 950 cGy irradiation only with no donor cells infused. All of the mice that died had clinical evidence of GVHD excluding the irradiation controls. All mice alive at the end of the experiment had no evidence of GVHD.

Fig 4. Effect of chloroquine treatment on development of GVHD after BMT (LP/J donor into C57BL/6 recipient). C57BL/6 recipient mice treated with chloroquine at 400 μg (or 200 μL PBS) IP for 5 days were irradiated (850 cGy), followed by infusion of 1 × 10⁸ LP/J bone marrow cells and additional spleen cells containing 2 × 10⁷ Thy 1 T cells. After transplantation, mice were injected with chloroquine at 400 μg IP once weekly or twice weekly, or PBS at 200 μL was administered twice weekly until the end of the experiment. Controls included C57BL/6-recipient mice transplanted with C57BL/6 donor cells and treated with chloroquine at 400 μg as described above (syngeneic controls) and mice which received 950 cGy irradiation only with no donor cells infused. All of the mice that died had clinical evidence of GVHD excluding the irradiation controls. All mice alive at the end of the experiment had no evidence of GVHD.

Chloroquine treatment had no effect on serum IL-2 levels. Serum IL-2 levels were evaluated in mice that had received PBS, 200 μg of chloroquine daily for 5 days, 400 μg of chloroquine daily for 5 days, or 400 μg chloroquine daily for 5 days followed by doses once or three times per week. Evaluation of serum IL-2 levels was performed 12 to 24 hours after the last IP dose (Table 2). No significant differences were observed for any of the treatment regimens, although serum IL-2 levels in mice treated with short-course chloroquine (400 μg daily for 5 days) were lower than that in PBS-treated mice (P = .15). Interferon-α levels were unmeasurable in any of the groups and could not be analyzed (data not shown). Thus, chloroquine treatment had no effect on MHC class I or II expression and had no significant effect on serum IL-2 levels.

Effect of chloroquine treatment on the ability of minor histocompatibility antigen-expressing targets to stimulate primed T cells. The effect of chloroquine on class II MHC presentation in vitro is reversible with resumption of normal antigen presentation within 24 hours after removal of chloroquine from the culture conditions. To evaluate the in vivo effect of chloroquine treatment on antigen presentation of MiHC in vitro, we found it necessary to fix cells immediately after the mouse as previously described. Glutaraldehyde fixation ensures that no further processing or presentation can occur and that all molecules on the cell surface do not change. We evaluated the ability of C57BL/6 cells to stimulate MiHC-reactive T cells using a well-described model, C3H.SW anti-C57BL/6. This model is H-2b-identical and incompatible for many MiHC with induction of T-cell proliferative responses after in vivo priming and GVHD.

Stimulator cells were C57BL/6 spleen cells from mice treated with PBS, chloroquine at 200 μg or 400 μg IP for 5 days with removal 4 hours after the last IP dose (Fig 4). The C57BL/6 spleen cells were immediately fixed by incubation with 0.05% glutaraldehyde for 30 seconds followed by incubation in a proliferation assay as stimulators. Responders were from C3H.SW (H-2b) mice previously primed with 2.5 × 10⁷ C57BL/6 (H-2b, MiHC-incompatible) spleen cells IP at least 6 weeks previously. C3H.SW anti-C57BL/6 responder cells were incubated at 5 × 10⁵ responder cells per well at various R:S ratios, with an R:S of 4:1 shown in Fig 5. We observed a significant decrease in either whole spleen cell or purified T-cell proliferative responses to MiHC in chloroquine-treated mice compared with that in PBS-treated controls (Fig 5). We saw no effect of chloroquine treatment on the ability of C57BL/6 glutaraldehyde-fixed stimulators to induce an alloreactive response by BALB/c T-cell responders (Fig 5; P < .05). We noted that the proliferative response induced in both BALB/c and C3H.SW anti-C57BL/6 responders by glutaraldehyde C57BL/6 cells was much lower (about 25%) as compared with that of unfixed irradiated stimulators. Thus, it appears that glutaraldehyde-fixed spleen stimulators from chloroquine-treated mice cannot
present MiHC antigens to stimulate previously primed MiHC-reactive T cells. It can be inferred that chloroquine treatment in vivo decreases antigen processing of MiHC in the context of class II MHC.

DISCUSSION

Chloroquine, an agent known to inhibit MHC class II processing and presentation of antigen to CD4+ T cells in vitro, was used to decrease MiHC presentation in vivo and to evaluate its effect on T-cell priming and GVHD. Using this approach, we found that treatment with chloroquine inhibits the proliferative and cytolytic T-cell priming responses to MiHC. The cytolytic response in the C57BL/6 anti-BALB.B response is a CD4+-dependent CD8+ T-cell response,14 and the lack of cytolytic activity is probably because of a lack of CD4+ T-cell priming and help. Treatment of recipient mice with chloroquine also significantly decreased GVHD. Because GVHD in the LP/J donor into C57BL/6 recipient model is very CD4+-dependent,7 we most likely are seeing a decrease in GVHD secondary to decreased activation of CD4+ donor T cells. Stimulator cells from chloroquine-treated mice, which were glutaraldehyde-fixed to ensure no reversal of chloroquine effect after incubation in vitro, were unable to stimulate MiHC-reactive T cells to respond to MiHC. This supports the hypothesis that chloroquine was suppressing class II MHC presentation of MiHC rather than this suppression being caused by any other mechanism. These findings are consistent with the observation that in vivo removal of B cells, a major class II MHC antigen-presenting population, resulted in decreased GVHD18 and are consistent with the demonstration that blockade of class II MHC ability to bind antigen by using a peptide with a high affinity for class II MHC results in a decrease in GVHD.

Previous approaches to control GVHD have focused on the manipulation of one of the effector cell populations in GVHD, such as T cells or NK cells. On the other hand, the modulation of the stimulating population such as class II MHC antigen-presenting cells represents an alternative approach to modulate GVHD, which warrants further investigation.

It is possible that chloroquine had immunosuppressive effects other than the suppression of class II MHC antigen presentation; therefore, we evaluated the effects of chloroquine treatment on potential factors that may affect T-cell responses in vivo. Chloroquine treatment had no effect on

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**Table 1. Effect of Chloroquine Treatment In Vivo on MHC Class I and II Expression in Spleen**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% la⁺ Cells (mean ± SEM)</th>
<th>MCF (mean ± SEM)</th>
<th>% D⁺ Cells (mean ± SEM)</th>
<th>MCF (mean ± SEM)</th>
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<td>55.5 ± 9.7</td>
<td>25.4 ± 9.7</td>
<td>99.6 ± 6</td>
<td>13.5 ± 2.5</td>
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<tr>
<td>Chloroquine (200 µg for 5 d)</td>
<td>54.5 ± 9.3</td>
<td>24.3 ± 9.3</td>
<td>99.1 ± 6</td>
<td>13.4 ± 2.3</td>
</tr>
<tr>
<td>Chloroquine (400 µg for 5 d)</td>
<td>56.5 ± 10.4</td>
<td>24.0 ± 10.4</td>
<td>97.2 ± 5.0</td>
<td>12.7 ± 5.0</td>
</tr>
<tr>
<td>Chloroquine (400 µg once/wk for 4 wk)</td>
<td>54.7 ± 9.7</td>
<td>19.4 ± 9.7</td>
<td>99.5 ± 6</td>
<td>14.9 ± 2.6</td>
</tr>
<tr>
<td>Chloroquine (400 µg 3 times/wk for 4 wk)</td>
<td>50.2 ± 8.0</td>
<td>23.8 ± 8.0</td>
<td>98.7 ± 6</td>
<td>13.7 ± 2.5</td>
</tr>
</tbody>
</table>

C57BL/6 mice received PBS, chloroquine at 200 µg or 400 µg IP daily for 5 days, or chloroquine at 400 µg IP daily for 5 days, followed by 400 µg either once or 3 times per week for 4 weeks before evaluation by immunophenotyping. Values are expressed as the percentage of spleen cells expressing either MHC class I (la⁺) or class II (D⁺) and as the MCF ± SEM. A minimum of 1,000 cells was counted in each assay. This experiment was repeated twice with identical results.

Abbreviation: MCF, mean channel fluorescence.

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**Table 2. Effect of Chloroquine Treatment on Serum IL-2 Levels**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum IL-2 Level (pg/mL)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>13.0 ± 5.4</td>
<td>.53</td>
</tr>
<tr>
<td>Chloroquine (200 µg for 5 d)</td>
<td>10.6 ± 3.8</td>
<td>.15</td>
</tr>
<tr>
<td>Chloroquine (400 µg for 5 d)</td>
<td>6.9 ± 3.7</td>
<td>.86</td>
</tr>
<tr>
<td>Chloroquine (400 µg for 5 d/400 µg 1/wk for 4 wk)</td>
<td>14.5 ± 2.2</td>
<td>.73</td>
</tr>
<tr>
<td>Chloroquine (400 µg for 5 d/400 µg 3/wk for 4 wk)</td>
<td>12.1 ± 1.8</td>
<td>.73</td>
</tr>
</tbody>
</table>

Mice were treated with PBS or with chloroquine at 200 µg, 400 µg IP daily for 5 days, or 400 µg IP for 5 days, followed by treatment either once or three times weekly for 4 weeks. Serum IL-2 levels were then evaluated by enzyme-linked immunosorbent assay. Values are expressed as the mean ± SEM. Statistical analysis was performed by the Fisher’s exact two-tailed test for unpaired samples.

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**Fig 5. Effect of in vivo chloroquine treatment on MiHC presentation by C57BL/6 cells to C3H.SW anti-C57BL/6 primed T cells.** Spleen cells from C3H.SW mice that had been primed greater than 6 weeks previously with 2.5 × 10⁶ viable spleen cells (C3H.SW anti-C57BL/6) or naive BALB/c T cells were used as responders in this assay. Stimulators were C57BL/6 spleen cells from mice receiving IP daily for 5 days with either PBS at 200 µL ( ), chloroquine at 200 µg IP ( ■ ), or chloroquine at 400 µg ( □ ). Stimulator cells were removed 4 hours after the last IP dose and immediately were fixed in 0.1% glutaraldehyde for 30 seconds, but were not irradiated. After fixation, the responder cells were washed and placed in a routine [³H]-thymidine incorporation assay. Irradiation was performed in addition to glutaraldehyde fixation in some groups (not shown) with no difference in results. This figure is representative of two experiments.

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T-cell responses to mitogens or to allogeneic stimulators or on the proportion of T cells present in the spleen. In addition, chloroquine treatment appeared to have no effect on B-cell mitogen responses or on B-cell numbers in the spleen. Contrary to the observations in other studies, we saw no effect on MHC class I or II expression in the spleen after chloroquine treatment. Evaluation of serum IL-2 levels also showed no significant effect on IL-2 levels in chloroquine-treated mice. Evaluation of other possible cytokines that have been affected by chloroquine in vitro (IL-1 and IL-6) and other possible effects of chloroquine treatment including inhibition of NK cell activity and Ig production were not in the scope of these investigations. Moreover, we felt that it is unlikely for these factors to be significant for T-cell priming to MHC or GVHD in these models. Thus, it appears that chloroquine treatment inhibits T-cell priming and GVHD secondary to MHC disparities by inhibition of MHC class II presentation of MHC. This mechanism of action is unique compared with those for other immunosuppressive agents (ie, cyclosporin A, FK-506) currently used to manage GVHD.

The ability of chloroquine to decrease MHC class II presentation is probably due to a combination of four potential mechanisms. With an increase in lysosomal and endosomal pH induced by chloroquine, the ability of MHC to complex with peptide is compromised. There also may be stabilization of the invariant chain association with either the α or β chain of the class II MHC molecule resulting in retardation of dissociation and peptide binding. In addition, the increased pH may affect the pattern of digestion of antigenic peptide by the lysosomal acid hydrolases. Finally, chloroquine appears to have an effect on the recycling of antigenic peptide association with surface class II MHC in vitro. Previous to this study, there are limited data which show that chloroquine decreases antigen presentation in vivo in mice.

Chloroquine's effect on class II MHC antigen-presenting cell function appears to be generalized on all antigen-presenting cell populations. Previous studies have evaluated the effect of chloroquine on macrophages, dendritic cells, and B cells in vitro and in vivo. The effect of chloroquine is similar in most studies. Class II MHC association with peptides in the late endosome are preferentially inhibited by chloroquine, and there is inhibition of class II MHC binding to certain antigen epitopes by chloroquine with normal presentation of other epitopes. Other studies have suggested that chloroquine has a selective inhibitory effect on the endogenous antigen presentation pathway and little effect on the exogenous class II MHC pathway. If this is true in vivo, then the effect of chloroquine may be relatively specific resulting in inhibition of T-cell responses to endogenous peptides that result in GVHD. On the other hand, T-cell responses to exogenous peptides (ie, viral antigens) will be unaffected. Also unknown is whether there is a differential effect on activation of CD4+ T-cell subpopulations. Delayed infusion of Th2 CD4+ T cells after BMT results in induction of T-cell tolerance and reduced GVHD, and it is possible that chloroquine inhibits Th1 CD4+ T-cell activation to MHC, thus preferentially allowing a preponderance of Th2 CD4+ T cells after BMT and induction of tolerance. Thus, the actual mechanism by which chloroquine decreases GVHD in vivo still requires further study.

Chloroquine (or hydroxychloroquine, a less toxic derivative) may potentially be useful for treatment of GVHD. Both chloroquine and hydroxychloroquine have similar therapeutic efficacy in various disease states, although hydroxychloroquine has less retinal toxicity. To our knowledge, there are no published studies using chloroquine treatment for GVHD. There is widespread use of hydroxychloroquine in patients with systemic lupus erythematosus and rheumatoid arthritis, with good efficacy. The response is only after prolonged therapy (>3 months), probably because of its mechanism of action through inhibition of MHC class II presentation resulting in decreased T-cell activation slowly over time. In this report, we used chloroquine prophylactically rather than therapeutically, because we hypothesized that an in vivo effect on primary CD4+ T-cell activation to MHC would be easier to show. It is possible that it may work in treatment of active GVHD rather than as a prophylaxis, but we would expect it to work only after prolonged administration. Recently, chloroquine has been shown to improve the outcome of children with interstitial lung disease. Because chronic GVHD-associated obstructive lung disease is a significant complication after alternate donor transplants, especially in children, chloroquine may be especially efficacious for this disease. It has been hypothesized that chloroquine treatment (by decreasing invariant chain dissociation from MHC class II) results in a competitive inhibition of low-affinity endogenously produced self peptides and that it is these peptides that are responsible for the development of autoimmune disease. It is possible that this is also true in GVHD.

Using an approach to inhibit T-cell priming and GVHD by inhibition of antigen presentation represents an approach that is unique and may have certain advantages. We feel that this approach should be explored further because we have recently seen that removal of B cells, an important MHC class II presenting cell population, also results in a decrease in T-cell activation in vivo and a decrease in GVHD. Use of a drug that has relatively low toxicity in vivo and can be titrated easily offers certain distinct advantages. In addition, chloroquine (or hydroxychloroquine) may be synergistic when used in combination with other immunosuppressive agents that suppress T cells directly, such as cyclosporin A.

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