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MD1 Expression Regulates Development of Regulatory T Cells

Reginald M Gorczynski,1* Yu Kai,* and Kensuke Miyake†

Intense interest has centered around the role of a subset of regulatory T cells, CD4+CD25+ Treg, in controlling the development of autoimmune disorders, allograft rejection, infection, malignancy, and allergy. We previously reported that MD1, a molecule known to be important in regulation of expression of RP105, also was important in regulating alloimmunity, and that blockade of expression of MD1 diminished graft rejection in vivo. One mechanism by which an MD1-RP105 complex exerts an effect on immune responses is through interference with an LPS-derived signal delivered through the CD14-MD-2-TLR4 complex. We show below that LPS signaling for Treg induction occurs at higher LPS thresholds that for effector T cell responses. In addition, blockade of MD1 functional activity in dendritic cells (using anti-MD1 mAbs, MD1 antisense deoxyoligonucleotides, or responder cells from mice with deletion of the MD1 gene), resulted in elevated Treg induction in response to allogeneic stimulation (in vivo or in vitro) in the presence of LPS. These data offer one mechanistic explanation for the augmented immunosuppression described following anti-MD1 treatment. The Journal of Immunology, 2006, 177: 1078–1084.

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1 is now accepted dogma that mammalian T cell-mediated immunity depends not only upon TCR triggering by Ag-MHC complexes in association with costimulatory signals but also needs some engagement, likely on APCs, of innate immune response receptors, which classically recognize microorganism-specific motifs (PAMPs)2 (1, 2). A predominant receptor-ligand complex is formed between the mammalian TLR4-MD2-CD14 complex and bacterial LPS (3). Evidence for a role for TLRs in the acquired immune responses goes beyond the acknowledged data demonstrating an important role for such receptor recognition in immunity to infectious diseases (see Refs. 3, 4), and reflects, in part at least, a role for inflammatory cytokines produced following TLR signaling in development of acquired immunity (5). The net signaling threshold following LPS activation of TLR4-MD2-expressing cells is now believed to be regulated by another molecular complex, RP105-MD1, also expressed on the surface of a number of cells, which acts as a competitive inhibitor to TLR4-MD2 complexes for LPS binding (6).

There is a growing body of evidence suggesting a role for immunoregulatory CD4+CD25+ T cells (Treg) in the suppression of allorejection studied in a number of models (7–9). Independent studies have suggested that not only effector T cells but also Tregs express TLR4, which can enhance their proliferation/survival (10). Earlier data from our group suggested that functional blockade of MD1 (thus permissively fostering TLR4-MD2 signaling) led to decreased graft rejection (11). Given the data above suggesting that MD1 might act to modulate signals mediated by TLR4 engagement (6), we have asked whether graft prolongation afforded by MD1 blockade reflected a differential sensitivity of Treg vs Teff to TLR4-LPS activation threshold for Treg indeed occurs, at the level of dendritic cells (DCs), at a higher concentration than for Teff, such that blockade of MD1 expression in DCs (thus augmenting TLR4 signals) promotes Treg induction, and in consequence inhibits allosensitization in vitro and in vivo.

Materials and Methods

Mice and tumor target cells

Six- to 8-wk-old female C57BL/6 (H-2b) mice were obtained from The Jackson Laboratory. BALB/c (H-2b) and C57BL/6J (H-2b) mice were obtained from Charles River Laboratories. MD1-null mice (MD1-KO) were a gift from K. Miyake (University of Tokyo, Japan) (12). All mice were housed five per cage in the animal care facility at the Toronto General Hospital (Toronto, Canada). Mice were allowed food and water ad libitum and were used at 8–12 wk of age.

The 72-h Con A-activated and 51Cr-labeled spleen blasts were used as targets in the cytotoxicity assays described below, except where stimulation was with BALB/c cells (when P815 tumor targets were used). All cultures were performed using α-MEM medium supplemented with 10% FCS, 5 mM HEPES, and 10−5 M 2-ME (αF10).

Abs and cytokine quantification

The following biotinylated mAbs were obtained from BD Pharmingen: anti-IFN-γ (XMG 1.2), anti-IL-2 (JE5-5H4), anti-IL-4 (24G2), and anti-IL-10 (XRC-1). Rabbit heteroantibodies to the various mouse cytokines, streptavidin–alkaline phosphatase, and recombinant cytokines (IL-2, IL-4, IL-10, and IFN-γ) were purchased from Cedarlane Laboratories. Cytokine levels in the supernatants of MLR cultures were measured in triplicate by ELISA, with standardization of all assays using the respective recombinant cytokines.

Anti-mouse thy1.2 (CD90), anti-CD8 (CT-CD8), FITC-anti-mouse H2-Ab, anti-H2-Kb, anti-CD11c, and anti-CD80/CD86 were obtained from Cedarlane Laboratories. The rat anti-mouse MD1 mAb used (SH2.47.1) was described in an earlier publication (13).

LPS, anti-sense deoxyoligonucleotides (ODNs), and primer sets for MD1 and GAPDH

LPS from Salmonella minnesota (Re 595) was obtained from Sigma-Aldrich. The sequences of the 17-mer phosphorothioate modified antisense ODN and a control random ODN for MD1, are as follows: antisense ODN, 5′-P (S)-AGGCCAGCUCCGACACC-3′; and random ODN, 5′-P (S)-CAGUCGGAACCGCGAG-3′.

These oligonucleotides were synthesized by Midland Certified Reagent as described elsewhere (11). Delivery of ODNs to DC in culture used the cationic lipid GS2888 cytofectin (14). Following 6 h of incubation in serum- and antibiotic-free α-MEM, the cells were incubated overnight in αF10. Independent studies in our laboratory using FITC-conjugated ODNs

*Departments of Surgery and Immunology, University Health Network and the Toronto Hospital, Toronto, Ontario, Canada; and †Department of Microbiology and Immunology, Division of Infectious Genetics, Institute of Medical Science, University of Tokyo, Tokyo, Japan

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1 Address correspondence and reprint requests to Dr. Reginald M. Gorczynski, Toronto Hospital, NU-G001, 200 Elizabeth Street, Toronto, Ontario, Canada M5G2C4. E-mail address: gorczynski@uhnres.toronto.ca

2 Abbreviations used in this paper: PAMP, microorganism-specific motif; Treg, regulatory T cell; DC, dendritic cell; ODN, deoxyoligonucleotide.
indicated >90% transfection efficiency using this technique (R. M. Gorczynski, unpublished data). Then, the cells were washed four times with PBS10 and used in the assays described. Treated mice received 100 μg of ODN per injection i.v. in 200 μl of saline (three injections at 60-h intervals) As assessed by real-time PCR analysis using mRNA isolated from splenocytes of treated mice 2 days after the third injection, suppression of MD1 in vivo using this approach was >5-fold (11).

FACS staining to determine the effect of in vitro in vivo incubation with ODNs to MD1 on cell surface expression of MHC, CD80, and CD86, used red cell-depleted (NH4Cl) spleen cell suspension, and incubation with FITC Abs (or isotype controls) for 60 min at 4°C.

**Mixed lymphocyte cultures**

Responder spleen T cells, enriched for T cells by passage over nylon wool (>90% CD90+ by FACS; Polysciences), were cultured in quadruplicate in 96-well microtiter plates (1.5 × 10⁷ responder and 3 × 10⁵ mitomycin-C-treated BALB/c stimulator DCs) in 250 μl of PBS10 per well. DCs were obtained by overnight incubation of plastic adherent spleen cells obtained from spleen cells pooled from three BALB/c donors. As determined by FACS, >90% staining for CD11c+ cells was seen following this enrichment procedure. In some experiments, DCs were derived from adherent cultures which had been pretreated (for 6 h) with ODNs before overnight incubation (see above). Fifty-microliter aliquots of the cell supernatants were removed at 40 h for cytokine analysis by ELISA (see above). Cells were harvested at 5 days from replicate wells, counted, and used at different E:T ratios for lysis of 51Cr-labeled P815 targets.

**Assessment of CTL activity**

Effector (E) cells obtained from 5-day MLCs were incubated with 2 × 10⁶ 51Cr-labeled targets (T) cells at various E:T ratios for 5 h at 37°C. Supernatants were collected and assayed for 51Cr release, with percent specific lysis calculated in standard fashion. Lytic efficiency in different groups was compared after calculation of LU/10⁵ recovered cells (1 LU considered that necessary to kill 25% of 2 × 10⁵ targets in 5 h).

**Isolation of Treg from MLC cultures**

CD4+ Treg were enriched at 72 h from MLC cultures set up as above, but also with the addition of exogenous IL-2 (100 U/ml) to all cultures, using a CellSep procedure described elsewhere (15). Two serial enrichments were performed with PE-coupled anti-CD4 mAb (>96% CD4+ cells obtained). Subsequent FACS analysis with FITC-anti-CD25 mAb showed that these cells were >90% CD4+CD25+. Foxp3 mRNA was examined by real-time PCR in these cells (below), and 3 × 10⁶ CD4+ cells were used to suppress a CTL/cytokine response in a fresh MLC culture.

**RT-PCR for Foxp3 mRNA**

RNA was extracted with TRIzol reagent from snap-frozen cultured cells, reverse transcribed, and real-time PCR used to quantitate mRNA for Foxp3 (standardized to a panel, including GAPDH and CypA as controls), using the following primer pairs, as described in an earlier report (16): GAPDH, (sense) TGGCAGATGATGACACTCAAGAG, (antisense) TGAAGTCGAGGAGACACCT; CypA (sense) TGGAGCCTGTAGCAGCAG, (antisense) TGAAGTGGTGAAGGCTTGGCAC; Foxp3, (sense) GTTTACTGGCATGTGTGCCCT, (antisense) CCACTCGCCACAAAGCAGTCG.

**Skin grafting and animal treatments**

Full-thickness tail-skin grafts were harvested from donor BALB/c mice and skin grafts (~1 cm²) transplanted to the lateral thoracic wall of groups of recipient mice anesthetized with Avertin (Aldrich). The grafts were covered with a double layer of gauze and plaster bandage for 7 days when the covering was removed. Grafts were inspected daily for signs of rejection by observers blinded to the individual groups. Where recipients were treated with saline, random or antisense ODN, or anti-MD1 or isotype control Abs (in all cases, 100 μg per mouse in 200 μl), animals received i.v. injections at 60-h intervals while grafts were intact.

**Statistical analysis**

Initial ANOVA and comparisons between means using paired Student’s t test were performed with Prism 3.0 statistical analysis software (GraphPad). All experiments described below have been repeated at least three times with essentially equivalent results.

**Results**

**Suppression of allostimulation in MLC by anti-MD1 (or ODNs) depends upon LPS stimulation**

Previous studies from our laboratory reported that functional blockade of MD1 expression, using anti-MD1 mAb or MD1-specific ODNs, suppressed MLCs in vitro using LPS-pretreated stimulator cells and prolonged graft survival in vivo (11). To explore the role for LPS stimulation on this phenomenon, the following study was performed: C57BL/6 spleen T cells (nymph wool enriched) were mixed with allogeneic splenic BALB/c DCs in the presence/absence of different concentrations of LPs (from 0.1 to 10 μg/ml). One group of cultures also contained anti-MD1 mAb (Fig. 1c). Alternate studies used DCs (a) or T cells (b) that were pretreated with random or antisense (to MD1) ODNs in culture, again with varying concentrations of LPS. Typical data from one of three such studies are shown assaying induction of CTL at day 5 (Fig. 1) or cytokine production at 40 h (Fig. 2).

These data confirm and extend the observations reported elsewhere (11, 17), indicating that both anti-MD1 mAb and antisense ODN to MD1 can suppress CTL induction in vitro in MLCs, and change polarization in cytokine production from IFN-γ (prototypic-type 1 cytokine) toward IL-4, a prototypic type-2 cytokine.

**FIGURE 1.** Modulation of suppression of CTL development in MLCs by anti-MD1 ODN (a and b) or mAb (c) in the presence of varying concentrations of LPS. All cultures received 1.5 × 10⁵ C57BL/6 responder spleen T cells and 3 × 10⁵ BALB/c DCs. In all panels, the different groups received varying concentrations of LPS in culture, from 0 to 10 μg/ml. In a and b, either stimulator DCs or responder T cells were pretreated with ODNs specific for MD1 (or random ODNs) before culture (see Materials and Methods). In c, cells were cultured throughout groups with either a rat anti-MD1 mAb (5 μg/ml) or an isotype control normal rat IgG2a at the same concentration. CTL were assayed at different E:T ratios on day 5 using 51Cr-labeled BALB/c spleen ConA blast target cells. Dose-response curves were used to compute LU/10⁵ effectors cells for each group. Groups significantly different (p < 0.05) from the control (no LPS) are highlighted (continuous line, enhanced relative to control; broken line: suppressed relative to control).
Suppression was seen only in the presence of concentrations of LPS >1 μg/ml and was dependent on treatment of stimulator DCs, not responder T cells, with ODNs specific for MD1 (compare a with b in Figs. 1 and 2). In our previous study, all studies with ODN-treated DCs had used cells incubated overnight with LPS following transfection with ODNs (11), while studies with mAbs routinely used LPS-activated spleen stimulator cells as APCs. At concentrations of LPS ≤1 μg/ml there was evidence for an increase in CTL induction, compared with controls without LPS, with or without anti-MD1 treatment (see Fig. 1).

**Induction of Treg in MLC by anti-MD1 (or ODNs) in presence of LPS stimulation**

The data in Figs. 1 and 2 above indicate that with LPS concentrations ≥1 μg/ml, both anti-MD1 ODNs and mAb suppressed MLC responses, as assayed by a decline in CTL induction and altered polarization in cytokine production. At lower LPS concentrations, a modest enhancement in CTL induction occurred (Fig. 1). Although TLR4 is expressed at the surface both of Treg (10) and APC (6, 11), MD1 is expressed only on the latter (6, 11). This explains for the restriction of the effect of elevated LPS concentrations to suppression of MLCs only when DCs, not responder T cells, were pretreated with ODNs specific for MD1 (Figs. 1 and 2). In light of data implying an antagonist effect of MD1 on LPS-TLR4 signaling (6), we hypothesized that Treg were induced both by LPS acting on Treg directly (10) as well as following an elevation of LPS concentrations achieved at the level of APC by inhibition of MD1 antagonism of TLR4 signaling by anti-MD1. The following study tested this hypothesis:

C57BL/6 nylon-wool enriched splenic T cells were stimulated in MLC with BALB/c DCs in bulk cultures in 10 ml flasks, using 25-cm² growth area flasks (15 × 10⁶ responder T cells and 5 × 10⁶ stimulator DCs) and IL-2 (100 U/ml). In different groups, anti-MD1 Ab (or control Ig) were added along with LPS as in Fig. 1. In the groups shown in Fig. 1, a and b, respectively, either stimulatory DCs (a) or responder T cells (b) were pretreated with antisense MD1-specific ODNs (or random ODNs) before culture. At 72 h, cells were recovered from cultures, and CD4⁺ cells (>95% CD4 positive) enriched by magnetic bead separation (see Materials and Methods). Equivalent numbers of cells were recovered from all groups (~1.5–1.8 × 10⁶). FACS staining showed >90% CD4⁺CD25⁺ cells in all populations. A total of 3 × 10⁵ cells were added in triplicate to separate MLC cultures (1.5 × 10⁶ C57BL/6 nylon-wool enriched responder T cells and 3 × 10⁵ BALB/c stimulator DCs), while 5 × 10⁵ cells were used for real-time PCR estimation of Foxp3 mRNA expression (normalized to control mRNAs and to Foxp3 expression in fresh CD4⁺ splenocytes, nominally ascribed a value of 1). Data for one of these three studies are shown in Fig. 3.

As judged both by Foxp3 mRNA expression and by suppression of MLC CTL induction, it is apparent that in the presence of

**FIGURE 2.** Modulation of cytokine production in MLCs by anti-MD1 ODN (a and b) or mAb (c) in cultures shown in Fig. 1, in the presence of varying concentrations of LPS. Cytokines were assayed by ELISA in supernatants harvested at 40 h. Only data for the prototypic type-1 cytokine (IFN-γ) or type-2 cytokine (IL-4) are shown for clarity. Data for IL-2 and IL-10 essentially paralleled these findings (see Ref. 10). Once again, groups significantly different (p < 0.05) from the control (no LPS) are highlighted (continuous line, enhanced relative to control; broken line, suppressed relative to control).

**FIGURE 3.** Augmented development of CD4⁺ Treg cells harvested at 72 h from MLCs in the presence of LPS and anti-MD1 ODNs (a and b) or mAb (c), as assayed by decreased LU/10⁶ recovered cells and induction of Foxp3 mRNA (real-time PCR). See text and previous figures for more details. a, middle, Stimulator DCs (responder T cells) treated with ODNs before induction of Treg. 3 × 10⁵ CD4⁺ cells obtained from primary C57BL/6 anti-BALB/c cultures incubated with anti-MD1 and LPS were added to fresh secondary BL/6 anti-BALB/c MLC cultures, and CTL asayed 5 days later. Control secondary cultures (far left) contained no added Treg; control Foxp3 expression represents that detected in fresh splenic CD4⁺ T cells (nominally ascribed the level 1.0). In other groups, Foxp3 mRNA was assayed in the added CD4⁺ cells obtained from primary cultures (with ODNs/mAb). Groups different (p < 0.05) from controls (no LPS with induction of Treg) are highlighted (continuous line, enhanced relative to control; broken line, suppressed relative to control).
anti-MD1 (Fig. 3c), or using DCs pretreated with MD1 specific ODNs (Fig. 3a), concentrations of LPS ≥ 1 μg/ml augment induction of functional Treg as defined by their ability to suppress MLC responses, or by expression of Foxp3 mRNA. Pretreatment of responder T cells rather than DCs with MD1 specific ODNs (Fig. 3b) led to no augmentation of Treg seen in the presence of 10 μg/ml LPS beyond that seen (as measured by Foxp3 expression) when T cells or DCs were treated using random ODNs (Fig. 3, a and b) or when control Ig was added throughout (see Fig. 3c). This is consistent with the absence of MD1 expression on T cells (6, 11), and with the evidence for TLR4 on Treg (10). Given the data in Fig. 1, suggesting that CTL induction is actually increased in MLCs at lower LPS concentrations ± anti-MD1, these data are consistent with the hypothesis that optimal LPS enhancement of Ag-induced Treg production occurs when both T cells are stimulated in the presence of high LPS concentrations, and the LPS stimulation of DCs simultaneously occurs in the presence of suppression of MD1 inhibition of TLR4 signaling in DCs (6).

**FIGURE 4.** Altered induction of CTL in MLCs using stimulator or responder cells of wild-type or MD1−/− mice (a and b, respectively), with BALB/c cells as corresponding responder/stimulator cells. All cultures were set up as before with varying concentrations of exogenous LPS. Targets in 51Cr assays were 72-h Con A spleen blasts homologous with the DC stimulator source (e.g., in panel a, MD1+/+ or −/− targets; in panel b, P815 targets). Groups suppressed relative to controls (no LPS) are shown by the broken line (p < 0.05).

**FIGURE 5.** Increased efficacy of induction of Treg by LPS using DCs from MD1−/− mice. Induction of Treg over 72 h with IL-2 (see Fig. 3) occurred in MLCs using stimulator/responder cells of wild-type or MD1−/− mice (a and b, respectively), with BALB/c cells as corresponding responder/stimulator cells, with cultures set up with varying concentrations of exogenous LPS. At 72 h, 3 × 10^5 CD4+ cells obtained from these cultures were added to fresh secondary MLCs (BALB/c anti-MD1 in a; MD1 anti-BALB/c in b) and CTL assayed with targets homologous with the stimulator DCs (see Fig. 4) 5 days later. Control secondary cultures (far left) contained no added Treg; control Foxp3 expression represents that detected in fresh splenetic CD4+ T cells (nominally ascribed the level 1.0). In other groups, Foxp3 mRNA was assayed in the added CD4+ cells obtained from primary cultures. Groups different (p < 0.05) from controls (no LPS) are highlighted (continuous line, enhanced relative to control; broken line, suppressed relative to control).

**Suppression of CTL development and induction of Treg in MLC of MD1−/− mice by LPS**

One interpretation of the data in Figs. 1–3 is that the suppression of CTL development and type-1 cytokine production in MLCs initiated at high LPS concentrations in the presence of MD1 blockade at the level of DCs is explained by increased development of Treg in such cultures. We next examined the effect of LPS on MLC reactivity and Treg development (assaying MLC suppression and Foxp3 expression as in Fig. 3) using DCs or responder T cells from MD1−/− mice, with responder/stimulator DCs in MLCs derived from BALB/c. These experiments were set up essentially as described in the previous figures, using MD1−/− and MD1+/+ littermate control mice obtained from K. Miyake (University of Tokyo, Japan). Data for one of three studies are shown in Figs. 4 and 5. In Figs. 4a and 5a, cultures used BALB/c responder T cells and MD1+/+ or MD1−/− as stimulator DCs, with homologous 72 h Con A spleen cells as targets. In Figs. 4b and 5b, MD1+/+ or MD1−/− were used as responder T cells, and BALB/c DCs as stimulator (with P815 as target cells). These data support those from experiments shown in the previous Figures. Whether assayed by suppression of CTL in MLCs (Fig. 4a), or by induction of Treg over 72 h in the presence of allostimulation and IL-2 (Fig. 5a), exogenous LPS at high concentration using DCs from MD1−/− mice produced effects different from those using DCs from MD1+/+ mice, but akin to those seen in the studies of Figs. 1–3 using treatment with anti-MD1 mAbs or MD1 ODNs. No reproducible MLC suppression occurred when only the responder T cell population was from MD1−/− mice (Fig. 4b), and Treg induction using such responder cells (assayed by Foxp3 expression) was not significantly different from that in cultures with 10 μg/ml LPS and control (+/+) DCs or responder T cells (Fig. 5). These data support the hypothesis that Treg, assayed by Foxp3 expression and suppression in MLCs, are optimally induced by MD1 blockade (preventing antagonism of TLR4-LPS interaction at the levels of DCs (6)), and high exogenous concentrations of LPS (potentially acting directly on TLR4+ Treg precursors (10)).

**Augmented induction of Treg by blocking MD1 occurs primarily at level of DCs**

The studies reported above were interpreted to indicate that the primary effect of MD1 blockade in the LPS augmentation of Treg development in vitro, and suppression of CTL induction, was at the level of stimulatory DCs, not the responding T cell population (although Treg are known to express TLR4 (10)). To explore this more directly we performed the following study. In studies in Fig. 6 we used DCs from MD1−/− mice, or C3H mice (∼ pretreatment of the latter DCs with anti-MD1 ODNs as in Figs. 1–3) and responder T cells from BALB/c mice. In a complementary study (Fig. 7), we used BALB/c DCs treated with MD1-specific ODNs.
as stimulator, but responder T cells from C3H (±MD1 ODN pre-treatment) or MD1−/− mice. Data shown are from one of two studies.

It is evident that suppression of CTL induction in MLCs, and effective induction of Treg, occurred only when LPS-stimulated DCs were obtained from ODN-treated BALB/c (Fig. 7) or MD1−/− mice, but not from C3H mice (regardless of their treatment with ODNs) (see Fig. 6). Regardless of the source of responder T cells, when ODN-treated BALB/c DCs were used as stimulator with high-dose LPS, suppression of CTL induction and efficient induction of Treg occurred (Fig. 7). Because C3H mice are unresponsive to LPS, we conclude that these data show that the important interaction of LPS in induction of Treg and suppression of CTL induction in these assays occurs at the level of DCs, not responder T cells.

LPS increases skin graft survival in MD1−/− mice and MD1+/+ mice receiving anti-MD1 ODN

We have shown previously that both anti-MD1 mAb and MD1 ODN can prolong xenograft and allograft survival in normal mice (11). Because the data above showed that in vitro the effects of MD1 blockade are realized optimally in the context of exogenous LPS administration, one explanation for the in vivo data is that because all studies were performed using animals housed and maintained in a conventional animal facility, the data reflect ongoing endotoxin exposure. To explore whether additional exogenous LPS administration would reveal further effects of MD1 blockade in vivo, we grafted both MD1+/+ mice (receiving anti-MD1 ODN) and MD1−/− mice with allogeneic (BALB/c) skin grafts, and subdivided the groups into animals subsequently receiving LPS (30 μg i.p. or not. Control groups were untreated MD1+/+ mice or MD1−/− mice with antisense ODN only. Data in Fig. 8 are pooled from four studies (totaling 18 mice per group).

FIGURE 6. LPS-stimulated DCs from MD1−/− mice, but not untreated or ODN-treated C3H DCs, augment Treg development in allo-MLCs in vitro. CTL development in MLCs (a) and induction of Treg (b) used BALB/c responder T cells and DCs from MD1−/− mice or C3H mice (untreated or treated with MD1-specific ODNs). Homologous 72-h ConA blast targets were used in 51Cr assays. Induction of Treg was assayed using either Foxp3 expression or the ability to suppress CTL responses in secondary MLCs (see previous figures). Groups different (p < 0.05) from controls (no LPS) are highlighted (continuous line, enhanced relative to controls; broken line, suppressed relative to controls).

FIGURE 7. Results from equivalent studies to those shown in Fig. 6, using ODN-treated DCs from BALB/c mice as stimulator cells, and responder T cells from MD1−/− mice, or C3H mice (with/without treatment with MD1 ODNs). P815 targets were used in 51Cr assays in these studies. a, Data for 5-day primary MLCs. b, Data harvesting CD4+ cells from MLCs of a, and assaying them as Treg in fresh MLCs using homologous responder T cells (to the primary cultures). Control cultures (far left, no Treg) showed equivalent responses for all responder groups (see panel a). Groups different (p < 0.05) from controls (no LPS) are highlighted (continuous line, enhanced relative to control; broken line, suppressed relative to control).

FIGURE 8. Augmented suppression of allogeneic (BALB/c) skin graft rejection by LPS in MD1−/− mice, or in wild-type (MD1+/+) mice receiving anti-MD1 ODN. Data are pooled from three studies with a total of 18 mice per group. In some groups, animals received 30 μg LPS i.p. on the day of transplantation. Where shown, MD1+/+ mice also received antisense MD1 ODN (100 μg per mouse (see Materials and Methods) i.v. every 60 h beginning on the day of transplantation and continued throughout the course of the study. *, different from control (untreated MD1+/+), Mann-Whitney U test; **, significantly different from equivalent group without LPS.
These data support our previous observations (11), and in addition, he data in Figs. 1–5. Increased survival was seen in MD1+/+ mice only when simultaneous infusion of anti-MD1 ODN was used (compare △ and ○ in Fig. 8). Additional LPS administration further increased survival in these animals, as indeed it did in MD1−/− mice (see groups denoted by × in Fig. 8). However, note that even untreated MD1−/− mice showed significantly longer graft survival, compared with their untreated MD1+/+ counterparts (compare ○ and □ in Fig. 8).

Evidence that LPS-induced increased graft survival in MD1−/− mice and MD1+/+ mice receiving anti-MD1 ODN is associated with increased presence of CD4+CD25+ Treg

Our final studies examined whether the increased graft survival seen in Fig. 8 in MD1−/− mice (or MD1+/+ mice treated with LPS and MD1 ODNs) was a result of augmented Treg induction after such treatment (see Figs. 3–7). Groups of three mice per group (MD1+/+ or MD1−/−) received BALB/c skin grafts as in Fig. 8, along with LPS (30 μg/mouse i.p.). MD1+/+ mice also received i.v. injections of MD1 ODNs (100 μg per mouse at 60-h intervals from the day of transplantation). Control +/− mice received skin grafts only. Mice were sacrificed 10 days after transplantation, and aliquots of spleen samples were snap-frozen for later quantitative assessment of Foxp3 mRNA expression. CD8-depleted cells were analyzed by FACS for expression of CD4+CD25+ cells. A total of 5 × 10⁶ CD4−-enriched cells (obtained using the CellSep procedure described for Fig. 3, with two serial enrichments using PE-coupled anti-CD4 mAb (15)) of these CD8-depleted populations were added in triplicate to fresh cultures containing 1.5 × 10⁵ responder nylon wool-enriched T cells and 5 × 10⁶ mitomycin-C-treated BALB/c DCs. CTL were assayed from these cultures at day 5. Data shown in Fig. 9 are from one of three experiments.

In mice showing the most prolonged skin graft survival (MD1+/+ receiving LPS and anti-MD1 ODNs, or MD1−/− mice receiving LPS), increased expression of CD4+CD25+ Foxp3+ cells were obtained in CD8-depleted populations, with reference to grafted control MD1+/+ mice. CD4+−-enriched cells from these mice suppressed CTL induced from MD1+/+ T cells stimulated over 5 days in vitro with BALB/c DCs, in equivalent fashion to that seen with cells precultured in vitro with LPS and MD1 blocking agents (Figs. 3–7).

Discussion

CD4+CD25+ Treg are implicated in the maintenance of peripheral tolerance to certain self Ags, as well as transplantation tolerance and indeed regulation of a number of immune responses (7, 8, 16, 18–20). A number of studies have suggested that the suppressive function of Tregs is critically dependent on the source of (imma-

ture) DCs (9, 15, 21, 22). Unlike unresponsiveness following anergy induction in T cells, which is reversed following TLR activation of DCs via a mechanism involving the potentiation of Treg responsiveness to IL-2 by cooperative effects of IL-6 and IL-1, proinflammatory cytokines do not overcome Treg-mediated suppression (21). In fact, recent data have shown that exposure of CD4+CD25+ T cells to LPS induces up-regulation of a number of activation markers, enhances their survival and/or proliferation in a manner independent of APC, and augments their suppressor efficiency some 10-fold (10).

TLR4 activation is not the only PAMP signal believed to be important for control of Treg number/function. A recent study also has shown that TLR2−/− mice are more resistant to disseminated Candida infection, with an impaired production of the cytokine IL-10, and a 2-fold diminution of the CD4+CD25+ Treg population (23). Additional in vitro studies confirmed that enhanced survival of Treg cells was induced by TLR2 agonists, and in animals with disseminated candidiasis, improved resistance was seen after depletion of Treg cells. The authors concluded that C. albicans induced immuno-

suppression through TLR2-derived signals, which mediated both increased IL-10 production and survival of Treg cells (23).

Activation of monocytes by LPS, resulting in the production of a number of inflammatory cytokines, has been exhaustively studied. Activation occurs through a series of steps, in which LPS binds first to LPS-binding protein in plasma and is delivered to the cell surface receptor CD14, before transfer to the transmembrane signaling receptor, TLR4, along with the accessory protein MD2 (24, 25). Subsequent activation occurs through a number of intra-
cellular signaling pathways including the IκB kinase–NF-κB pathway and three MAPK pathways: ERK 1 and 2, JNK, and p38 (26). These various signaling pathways in turn activate a number of transcription factors that include NF-κB, AP-1, and AP-1 (cFos/c-Jun), which coordinate the induction of many genes encoding inflammatory mediators (27). As noted above, at the cell surface, TLR4 associates with MD2 as well as CD14. More recent data also have uncovered evidence for a JNK-interacting protein 3, as a TLR4-associated protein (binding through its NH2-terminal region), with evidence that in HEK 293 cells exogenously expressing TLR4, MD2, and CD14, coexpression of JNK-interacting protein 3 increased the complex formation of TLR4-JNK and LPS-

mediated JNK activation (28).

The domains of TLR4 and MD2 responsible for their interaction have been defined (29, 30). Consistent with a key role for MD2 in TLR4 signaling, MD-2−/− mice do not respond to LPS and survive endotoxic shock (12). In MD-2+/− embryonic fibroblasts, TLR4 was unable to reach the plasma membrane, residing predominantly in the Golgi apparatus, unlike its distribution at the leading edge surface of cells in wild-type embryonic fibroblasts, suggesting that MD-2 is es-

sential for the correct intracellular distribution and LPS-recognition of TLR4 (12). Other signals, at least in APC, are now known to regulate expression of TLR4-MD2 at the cell surface, including CD40. Thus, CD40 stimulation using cross-linking anti-CD40 Ab was shown to up-regulate TLR4-MD2 surface expression on exvivo-cultured
splenic DC, with subsequent enhanced IL-12p70 secretion after LPS restimulation (31). Regulation of TLR4 expression on activated T cells is less understood.

Because activation of TLR signaling by PAMPs is critical to the induction of immune responses and also is important in control of Treg development/proliferation, it might be anticipated that a tight regulation of such inflammatory stimuli would exist. Several years ago, Miyake et al. (32) reported that RP105 was a TLR homolog, lacking a signaling domain, and predominantly expressed on B cells. Surface expression of RP105 was stabilized by association with MD1, and the RP105-MD1 complex was reported to be important for LPS signaling in B cells. More recent data suggest that RP105 expression is much more widespread, paralleling that of TLR4 on APCs, a finding consistent with that reported earlier by our own laboratory (11). Miyake et al. (32) showed that the RP105-MD1 complex, acting through the extracellular domain of RP105, regulates TLR4 signaling in conventional APCs, likely via direct inhibition of LPS-TLR4 binding (6, 12). We showed several years ago (11, 33) that blockade of functional expression of MD1, using mAb or anti-sense deoxyoligonucleotides, inhibited allo- sensitization in vitro using LPS-activated stimulator cells, prolonged allograft and xenograft survival, and modulated fetal loss syndrome. This effect was seen following Ag presentation by both the direct and indirect recognition pathway (17). Independent data have confirmed that MD1 expression promotes graft rejection (34).

The data describe above provide evidence that this regulation of alloreactivity by MD1 can be understood in terms of a preferential stimulation of Treg by DCs activated by LPS along with suppression of MD1 antagonism of TLR4 signaling. Under conventional conditions (low-dose LPS with endogenous MD1 expression inhibiting LPS-mediated TLR4 signaling), the TLR4 signaling threshold is sufficient to activate only Teff (Figs. 1 and 2). At higher LPS concentrations, particularly in the absence of functional MD1 expression by stimulatory DCs (using DCs from MD1−/− mice, or from MD1+/− mice treated with anti-MD1 mAbs or anti-MD1 ODNs), the activation threshold for Treg is surpassed, and a net dominant stimulation of Treg occurs, resulting in increased graft survival and suppressed alloimmunity (see Figs. 3–9). This effect is not seen using DCs from TLR4 nonresponder C3H/HeJ mice but is independent of TLR4 expression on responder T cells (see Fig. 7). We hypothesize that the suppression of MLC reaction and induction of Treg occurs both by direct TLR4 signaling of Treg at high LPS concentrations (10), along altered DC costimulation that follows MD1 blockade in DCs. The possibility that an MD1-mediated modulation of LPS-triggering of Treg occurs in models of autoimmune disease (e.g., a collagen-induced arthritis model in mice) remains to be investigated.

Disclosures

The authors have no financial interest of conflict.

References