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FcγRI is required for TGFβ2-treated macrophage-induced tolerance

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Abstract

Macrophages treated with TGFβ2 (TGFβ2-Mφ) and antigen are highly tolerogenic *in vivo*, and induce antigen-specific and long-lasting tolerance in both naïve and primed mice via induction of suppressor/regulatory T cells. In this study, we examined the molecular pathways, including the requirements for Smad-dependent signaling, that are involved in the induction and function of tolerogenic TGFβ2-Mφ. Treatment of murine macrophages with TGFβ2 induced translocation of Smad2/3 to the nucleus, and impairment of Smad3-, but not Smad2-, dependent signaling inhibited the tolerogenic function of a TGFβ2-treated murine macrophage cell line. Gene expression in murine macrophages treated with TGFβ2 was evaluated by microarray analysis. The FcγRI gene was one of a number of immune-related genes differentially expressed in TGFβ2-Mφ, and appeared to be critical for tolerance in this system, since TGFβ2-Mφ from FcγRI deficient mice were unable to induce tolerance. The role that FcγRI plays in TGFβ2-Mφ-mediated tolerance is currently unclear. The results of this study provide important information about the factors that are critical for the induction of TGFβ2-Mφ-mediated tolerance, and a better understanding of these mechanisms could lead to the development of more effective tolerance-inducing strategies for the treatment of autoimmune/inflammatory diseases.

Keywords

Tolerance; macrophages; antigen presenting cells; TGFβ; FcγR; delayed type hypersensitivity; mice

Introduction

Tolerance induced by intraocular injection of antigen (i.e., anterior chamber associated immune deviation, ACAID) is associated with systemic and antigen-specific suppression of pro-inflammatory T cells that is characterized by altered cytokine production and impaired ability to mediate the delay type hypersensitivity (DTH) response (D'Orazio and Niederkorn, 1998; Kosiewicz et al., 1998). In ACAID, it appears that F4/80⁺ Class II⁺ cells

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residing in the anterior chamber of the eye are responsible for transporting the injected antigen to the spleen, where antigen-specific CD4 and CD8 regulatory cells capable of downregulating a peripheral pro-inflammatory T cell response are induced (Streilein and Niederkorn, 1985; Wilbanks and Streilein, 1990). This form of tolerance can be reproduced by intravenous injection of adherent peritoneal exudate cells (PEC), i.e., F4/80⁺ macrophages, that have been incubated overnight with soluble protein antigen and either fluid from the anterior chamber of the eye (aqueous humor) or supernatants from cultured ocular tissue (Kosiewicz et al., 1994; Wilbanks et al., 1992). Although a number of immunomodulatory factors have been found in aqueous humor and supernatants from cultures of ocular tissue (Cousins et al., 1991; Granstein et al., 1990; Taylor et al., 1992; Taylor et al., 1994), transforming growth factor β 2 (TGF β 2) has been identified as the critical factor that confers the tolerance-inducing activity on the PEC (Wilbanks et al., 1992) (Takeuchi et al., 1997). Similar to ACAID, substantial evidence suggests that these TGF β 2-treated macrophages (TGF β 2-M ϕ) mediate tolerance *in vivo* through the induction of functionally distinct CD4 and CD8 regulatory T cell populations in the spleen (Wilbanks and Streilein, 1990; Streilein, 1987; Alard et al., 2004; Kosiewicz et al., 2004).

The TGF β 2-M ϕ produce a variety of factors, including thrombospondin, that are thought to be involved in their tolerogenic function (Masli et al., 2002; Masli et al., 2006). However, our understanding of the pathways and molecules involved in the induction and function of these tolerance-inducing cells is still incomplete. In this study, we examined the role that (Smad-dependent) TGF β signaling plays in the induction of tolerogenic TGF β 2-M ϕ and the effect that TGF β 2 has on gene expression in these cells. We found that tolerance mediated by TGF β 2-M ϕ requires TGF β -induced signaling that is Smad3-dependent, and TGF β 2 induces expression of a variety of immune-related genes, including Fc γ RI, that appear to play an important role in the induction and/or mediation of tolerance in this system.

Materials and Methods

Mice

Six-8-week-old female BALB/cByJ, and C.129P2(B6)-*Fc ϵ RI^{tm1Rav}*N12 (BALB/cByJ background; deficient for gamma chain subunit; (Takai et al., 1994) mice were purchased from Taconic (Germantown, NY). Tribromoethanol was used as an anesthetic and was administered i.p. Mice were maintained under specific pathogen-free conditions following guidelines stipulated by the University of Louisville Institutional Animal Care and Use Committee.

Reagents

The following antibodies were used for flow cytometry, FITC-anti-mouse I-A (BD Biosciences, San Diego, CA) and PE-anti-mouse F4/80 (Caltag-In Vitrogen, Carmarillo, CA) antibodies, and for western blot analysis, anti-Smad2/3 (BD Biosciences or Upstate Biotechnologies) or anti- β -actin (Sigma) or anti-LSD1 as primary antibodies and HRP-conjugated (Zymed) secondary antibody. The following reagents were used for confocal microscopy, anti-Smad2/3 (Cell Signaling) and Alexa488-anti-rabbit IgG (In Vitrogen) and DRAQ5 (Alexis Biochemical). Ovalbumin (OVA; Sigma) was used as soluble protein antigen after passage through two Acticlean Etox chromatography columns (Sterogene, Carlsbad, CA) to remove endotoxin. LPS-low (15 EU/ml) OVA was then lyophilized and stored at -80°C.

Generation of TGF β 2-M ϕ

Peritoneal exudate cells (PEC) were generated by intraperitoneal injection of 2 ml of 3% thioglycolate (Sigma), 4 days before harvest. One million PEC/ml were cultured in serum-

free media comprised of RPMI 1640, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate and ITS supplement [1 mg/ml iron-free transferrin, 10 ng/ml linoleic acid, 0.3 ng/ml Na₂Se, and 0.2 mg/ml Fe(NO₃)₃; Collaborative Biomedical Products, Bedford, MA], TGFβ₂ (5 ng; R & D Systems, Minneapolis, MN), and LPS-low OVA (5 mg/ml) in 24-well plates overnight. Non-adherent cells were removed, and adherent cells gently harvested after incubation on ice for 1 hr using a cell-lifter. Cells were washed 3x in HBSS, and analyzed by flow cytometry before use in all experiments (>90% of adherent PEC were consistently found to be F4/80⁺; see Fig. 1). Cells treated in this manner are referred to as TGFβ₂-Mφ in the text and Figures. Control cells (Mφ) were cultured under the same conditions, but in the absence of TGFβ₂.

Cell lysis and western blot

For cytosolic protein extraction, cells were washed with PBS and resuspended in lysis buffer (10 mM Tris-HCl, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and 0.5 mM DTT) containing Phosphatase Inhibitor Cocktail 2 (Sigma) and protease inhibitor cocktail (Sigma), and supernatants collected following centrifugation. For nuclear protein extraction, the pellet was then incubated for 1 hr in nuclei lysis buffer (25 mM Tris-HCl, 5% TritonX-100, 1% deoxycholate, 0.35 M NaCl and 10 mM EDTA) containing phosphatase inhibitor. The supernatants were collected and stored at -80°C until assayed. Protein concentration was measured using the BCA protein assay kit (Pierce). Thirty micrograms of total cellular protein was suspended in lithium dodecyl sulfate (LDS) buffer, heated for 10 min at 70°C, and loaded onto NuPAGE® Novex 10% Bis-Tris Gels (Invitrogen) and then transferred to PVDF membranes (Invitrogen). Visualization of the bands was performed using the ECL detection kit (GE healthcare) and densitometric analysis was performed using Bio-Rad Fluor-S MultiImager/FX phosphorimager systems. The membrane was stained with Fast Green to confirm that the same amount of protein was loaded.

Confocal Microscopy

For confocal microscopy, cells were incubated overnight on coverslips, fixed in 4% formaldehyde/PBS for 1 hr at room temperature then permeabilized with TritonX-100. Samples were incubated with anti-Smad2/3 antibody overnight at 4°C followed by Alexa488-anti-rabbit IgG antibody for 1 hr at room temperature, and DRAQ5 for 15 mins. Samples were analyzed by confocal microscopy (Olympus FV1000).

Transfection of macrophage cell line

Cells of the macrophage hybridoma cell line, Mφ59 (Liu et al., 1985), were stably transfected with dominant-negative mutants of Smad2 and/or Smad3. The coding regions of the dominant negative (DN) Smad2 and Smad3 mutants were sub-cloned from the original constructs, pCMV-Smad 2 and pCMV-Smad 3 (Warner et al., 2003; Warner et al., 2003), into pcDNA3.1/Zeo (Invitrogen; Smad2-DN) and pcDNA3.1 (Smad3-DN), respectively. The Mφ59 cells were transfected with 1 µg of Smad2-DN and/or Smad3-DN using lipofectamine (Invitrogen) according to the manufacturer's instructions, and cells that were stably transfected with Smad2-DN and/or Smad3-DN were selected by addition of Zeocin and/or neomycin to the culture media.

Delayed type hypersensitivity (DTH) assay to test for tolerance

Four thousand antigen (LPS-low OVA)-pulsed TGFβ₂-treated PEC (TGFβ₂-Mφ) were injected i.v. into the tail-vein of mice. Seven days later, TGFβ₂-Mφ-injected mice were immunized s.c. with 100 µl of an emulsification of OVA (100 µg) and complete Freund's adjuvant [CFA; incomplete Freund's adjuvant (Sigma) and 3.3 mg/ml of desiccated *M.*

tuberculosis, H37 RA (Difco Labs, Detroit, MI)]. Seven-fourteen days after immunization, mice were challenged in the right ear pinnae with an intradermal injection of OVA-pulsed PEC (1×10^5 PEC/10 μ l of HBSS). Ear swelling as an indicator of an DTH response was measured 24 and 48 hrs later using a micrometer (Mitutoyo, MTI Corp., Paramus, NJ). The negative controls received an ear challenge only.

Microarray analysis

Total RNA was isolated using the UltraspecTM RNA reagent (Biotech Laboratories) according to the manufacturer's instructions. The mRNA was isolated from total RNA using the Oligotex mRNA kit (Qiagen) according to the manufacturer's instructions. The (ds)-cDNA was synthesized using the Superscript Choice system (Gibco BRL Life Technologies) and T7-(dT)₂₄ primers, and was hybridized on a microarray genechip containing probe sets interrogating approximately 36,000 full-length mouse genes and EST clusters from the UniGene data base (Affymetrix). Data are presented (Table 1) as fold differences between control and TGF β -M ϕ , and reported differences were found at least twice. (NCBI accession #: GSE45382)

Realtime PCR

Total RNA was extracted using the Picopure RNA isolation kit (Arcturus, Mountain View, CA), and reverse transcribed using the Taq Man reverse transcriptase kit (Applied Biosystems, Foster City, CA). The cDNA was amplified in duplicate by real-time PCR using the SYBR Green PCR kit (Applied Biosystems, Foster City, CA) with primers (300 nM) for GAPDH and Fc γ RI. Fc γ RI mRNA levels were normalized relative to GAPDH mRNA expression. Data are presented as the fold-change relative to untreated cells. Primer pairs were designed using software provided by Applied Biosystems, and synthesized and purified by HPLC by IDT (Coralville, IA). Primer pairs are as follows: GAPDH, 5'-GGAGCGAGACCCCACTAACA-3', 5'-ACATACTCAGCACCGGCCTC-3'; Fc γ RI, 5'-CGATGGCGTGTATGAAGAAGTAAC-3', 5'-CCATCGGGCGCTTCTTT-3'.

Statistical analyses

Data were subjected to analysis by ANOVA and the Tukey-Kramer multiple comparisons test and/or the student's *t* test. A value of $p < 0.05$ was considered significantly different. All experiments were performed at least twice.

Results

TGF β 2 induces translocation of Smads

Macrophages treated with TGF β 2 and pulsed with antigen (i.e., TGF β 2-M ϕ) *in vitro* induce long-lasting antigen-specific tolerance when injected i.v. into either naïve or immunized mice (Kosiewicz et al., 1994; Wilbanks et al., 1992). Because TGF β , an immunosuppressive cytokine, can bind to proteins either expressed by or bound to the surface of macrophages (Yehualaeshet et al., 1999), there is a possibility that TGF β 2-M ϕ simply serve as transporters of TGF β that adheres to their surface. Alternatively, TGF β 2 likely induces tolerogenic function directly in macrophages by modulating target gene expression via an TGF β signaling pathway. Most immune-related TGF β signaling is mediated via the Smad-dependent pathway, and for this reason, the following analysis of Smad involvement was performed. Initial experiments were performed to determine whether treatment of macrophages with TGF β 2 induces nuclear translocation of Smads (an indicator of TGF β signaling). Nuclear and cytosolic extracts were prepared from TGF β 2-M ϕ or control M ϕ , and evaluated by western blot. As shown in Figure 2, treatment with TGF β 2 induces translocation of Smad2/3 from the cytoplasm to the nucleus in TGF β 2-M ϕ as indicated by a

decrease in cytosolic, and a concurrent increase in nuclear, Smad2/3. Similar results were found using the murine macrophage hybridoma cell line, M ϕ 59, treated with TGF β 2 (TGF β 2-M ϕ 59; Fig. 3). Translocation of Smad2/3 from the cytoplasm to the nucleus was confirmed by confocal microscopy and was indicated by the accumulation of Smad2/3 in the nucleus and a concomitant decrease in the cytosol (Fig. 2). These data suggested that treatment of macrophages with TGF β 2 under conditions that generate tolerogenic TGF β 2-M ϕ induces signaling via the Smad-dependent pathway, and therefore, very likely modulation of target gene expression that influences the function of these cells.

Smad3, but not Smad2, dependent signaling is required for tolerogenic function in TGF β 2-M ϕ

Although the data shown in Figures 2 & 3 indicated that the Smad-dependent TGF β signaling pathway is activated in TGF β 2-M ϕ , these experiments did not address whether TGF β signaling is actually required for the tolerogenic function exhibited by TGF β 2-M ϕ . In the following experiments, the requirement for Smad-dependent TGF β signaling for tolerogenic function in TGF β 2-M ϕ was evaluated by impairing Smad2 and/or Smad3 activity in the macrophage hybridoma line, M ϕ 59 (see Figure 3) and testing for tolerogenic activity *in vivo*. The M ϕ 59 cell line was used because it is much easier to transfect than primary macrophages, and previous studies have shown that M ϕ 59 cells cultured with antigen and TGF β 2 (TGF β 2-M ϕ 59) and TGF β 2-M ϕ exhibit comparable tolerogenic properties *in vivo* (Hara et al., 1992). In these experiments, M ϕ 59 cells that had been stably transfected with the dominant negative constructs, Smad2-DN, Smad3-DN, or both Smad2-DN and Smad3-DN, were cultured with OVA and TGF β 2 overnight then injected i.v. into mice. Seven days later, mice were immunized with OVA and CFA, and the DTH response measured on Day 10–14 after immunization. As expected, the DTH response in mice that served as tolerance controls (Fig. 4, third column; i.e., mice that had been treated with TGF β 2-M ϕ 59 cells prior to immunization) was significantly lower than in the positive controls (Fig. 4, second column; i.e., mice that had been immunized only). Interestingly, TGF β 2-M ϕ 59 cells that had been stably transfected with either the Smad3-DN (Fig. 4, fifth column) or both Smad2-DN and Smad3-DN (Fig. 4, sixth column) constructs failed to suppress the DTH response (i.e., failed to mediate tolerance). However, TGF β 2-M ϕ 59 cells stably transfected with the Smad2-DN construct were as effective as the TGF β 2-M ϕ 59 tolerance controls (Fig. 4, third column) in suppressing the DTH response (Fig. 4, fourth column). Taken together, these data indicate that the induction of tolerogenic properties in TGF β 2-M ϕ requires the Smad-dependent TGF β signaling pathway and involves Smad3.

Gene expression in TGF β 2-M ϕ cells

To evaluate the effect of TGF β 2 on gene expression in TGF β 2-M ϕ , microarray analysis was performed on cDNA from control M ϕ or TGF β 2-M ϕ (primary cells). Differentially expressed genes were identified as those exhibiting a 2-fold change in response to treatment with TGF β 2. A total of 66 unique genes were differentially expressed in TGF β 2-M ϕ vs control M ϕ , and of those, 47 were upregulated and 19 repressed. The primary responses fell into a number of functional categories including immune-related, extracellular matrix, transcriptional regulators, lipid metabolism and proteases/inhibitors (Table 1). Other responses were associated with cell cycling, cytoskeleton, and angiogenesis. Since the goal of this study was to understand the mechanisms involved in TGF β 2-M ϕ -mediated tolerance, our analysis focused on the genes related to immune function. Among the immune/chemotaxis-related genes that were differentially expressed between control and TGF β 2-M ϕ , 11 were up-regulated and 11 were repressed (Table 1). In agreement with a previous study (Masli et al., 2002; Masli et al., 2006), thrombospondin 1 expression by TGF β 2-M ϕ in the present study was upregulated approximately 3.5-fold and represented the greatest fold-change in gene expression among the immune-related genes. The subunits of the

complement component C1q (C1QA, C1QB, C1QC), Fc γ RI, haptoglobin, hepatocyte growth factor, IL-17 receptor, and leukotriene C4 synthase were also upregulated. A number of chemokine genes (CXCL1, CXCL2, CXCL5, CXCL6, CXCL9 and CXCL13) as well as other immune-related genes, including CD200, IL1Ra, protein tyrosine phosphatase, and C-type lectin were repressed (Table 1). These data indicated that TGF β 2 clearly modulates gene expression associated with immune function in TGF β 2-M ϕ .

Fc γ RI is required for TGF β 2-M ϕ induced tolerance

Among the immune-related genes that were upregulated in TGF β 2-M ϕ , *Fc γ RI* (Fc γ RI; CD64) was of particular interest for several reasons. First, activation of this high affinity Fc receptor in macrophages has been reported to elicit production of IL-10 (Sutterwala et al., 1998), and IL-10 has been shown to be involved in TGF β 2-M ϕ -induced tolerance (D'Orazio and Niederkorn, 1998). Furthermore, TGF β 2-M ϕ are thought to somehow “hand-off” antigen to B cells, and Fc γ RI could play a role in this process (D'Orazio et al., 2001; D'Orazio and Niederkorn, 1998; Niederkorn and Mayhew, 1995). The microarray analysis showed that *Fc γ RI* expression was >2-fold higher in TGF β 2-M ϕ than control M ϕ (Table 1), and this relative increase in *Fc γ RI* expression in TGF β 2-M ϕ versus control M ϕ was confirmed by quantitative real-time PCR (Fig. 5A).

To determine whether Fc γ RI plays a role in TGF β 2-M ϕ -induced tolerance, PEC were collected from wild-type or Fc γ RI -deficient mice, treated with OVA and TGF β 2 overnight (TGF β 2-M ϕ), then adherent cells injected into wild-type recipient mice. Seven days later, mice were immunized with OVA and CFA, and 10–14 days later the DTH response was evaluated. As shown in Figure 5B, TGF β 2-M ϕ from wild-type mice suppressed the DTH response (column 3) as expected. However, TGF β 2-M ϕ from Fc γ RI -deficient mice were unable to suppress an DTH response (Fig. 5B, column 4), suggesting that Fc γ RI plays a role in TGF β 2-M ϕ -mediated tolerance.

Discussion

Intravenous administration of antigen-pulsed TGF β 2-M ϕ elicits a complex cascade of events that involves a variety of immune cells as well as membrane-bound and secreted molecules, and ultimately leads to the induction of antigen-specific tolerance (Kosiewicz and Alard, 2004). Although we did not evaluate other TGF β isoforms (TGF β 1, TGF β 3) we anticipate that the effects would be the same, since all of the TGF β isoforms have very similar biological activity. The results described here provide important information about the early events in this process. We have found that TGF β 2 activates the Smad-dependent signaling pathway, and utilizes Smad3, and not Smad2, to induce the tolerogenic phenotype in TGF β 2-M ϕ . To the best of our knowledge, this is the first study showing unequivocally that the Smad-dependent TGF β signaling pathway is required for induction of the tolerogenic phenotype that characterizes TGF β 2-M ϕ . Although these data do not rule out the possibility that TGF β 2-M ϕ may also act by physically transporting TGF β bound to their surface to sites where TGF β can have a direct impact on the immune response, TGF β -induced signaling in these cells is clearly a requirement.

In response to treatment with TGF β 2, TGF β 2-M ϕ exhibited changes in the expression of a number of immune-related genes including upregulation of thrombospondin, C1q (complement) components, haptoglobin, FcR γ (CD64), integrin β 5, hepatocyte growth factor, IL-17R, leukotriene C4 synthase and down-regulation of CD200, IL1Ra, protein tyrosine phosphatase, C-type lectin, and the chemokines, CXCL1, CXCL2 (MIP2 α), CXCL5, CXCL13 (BLC), CCL6 (C10), CCL9 (MIP- γ /MRP-2) expression. Using differential display analysis, Masli and colleagues previously found that thrombospondin expression was increased in TGF β 2-M ϕ 59 cells and was important for their tolerogenic

function (Masli et al., 2002). In our current study, which involved a more comprehensive analysis of gene expression (i.e., microarray vs differential display analyses) in primary cells, we also found that thrombospondin expression was increased in response to TGF β 2. In contrast to mouse macrophages, mature human macrophages are not responsive to TGF β 1 unless differentiated in the presence of factors such as glucocorticoids, that up-regulate surface expression of TGF β RII (Gratchev et al., 2008). In combination with IL-4 and glucocorticoids, TGF β 1 elicits a gene expression program in human macrophages that resembles that of macrophages infiltrating atherosclerotic lesions (Gratchev et al., 2008). This differs from the gene expression profile that we have found in murine TGF β 2-M ϕ . Interestingly, we have found that Fc γ RI (CD64) was not only upregulated in response to TGF β 2, but was required for TGF β 2-M ϕ -mediated tolerance induction. The Fc γ RI molecule exhibits several interesting features, one or more of which could play a role in the mechanisms that are involved in TGF β 2-M ϕ -mediated tolerance induction. First, the ligation of Fc γ RI in macrophages can trigger the production of IL-10 (Sutterwala et al., 1998), and IL-10 has been associated with the induction of tolerance in this system (D'Orazio and Niederkorn, 1998). Therefore, ligation of upregulated Fc γ RI in TGF β 2-M ϕ could drive IL-10 production in this system *in vivo*. Secondly, antigen bound to Fc γ RI via antibody can be shunted to the Class I as well as the Class II pathways in monocytes and, therefore, efficiently presented to CD8 or CD4 cells, respectively (Guyre et al., 2001). Both CD4 and CD8 regulatory T cells are generated in TGF β 2-M ϕ -induced tolerance, and since these T cells appear to directly mediate the antigen-specific tolerance in this system, mechanisms that facilitate antigen processing and presentation by Class I and Class II to these cells could be critical for their induction (Wilbanks and Streilein, 1990; Streilein, 1987; Alard et al., 2004; Kosiewicz et al., 2004). Moreover, Fc γ RI is the only Fc receptor that can bind monomeric IgG with high affinity (Ioan-Facsinay et al., 2002), and the binding of monomeric IgG to Fc γ RI can trigger internalization in the absence of crosslinking in monocytes, suggesting that high Fc γ RI expression by TGF β 2-M ϕ may circumvent the need for high concentrations of antigen-specific antibodies (Harrison et al., 1994). Finally, antigen-specific antibody can also stably bind to Fc γ RI expressed on F4/80⁺ cells for long periods of time (Guyre et al., 2001). Several studies have suggested that B cells participate in TGF β 2-M ϕ -mediated tolerance, and some data suggest that the antigen-pulsed TGF β 2-M ϕ may actually “hand-off” the antigen to B cells via some currently unknown mechanism and a collaboration between the TGF β 2-M ϕ and B cells may be required for the induction of the regulatory T cells (D'Orazio and Niederkorn, 1998; Niederkorn and Mayhew, 1995). It is, therefore, possible that Fc γ RI expressed by the TGF β 2-M ϕ is somehow involved in this “handing-off” process. There are, therefore, at least three non-mutually exclusive mechanisms by which Fc γ RI could be involved in TGF β 2-M ϕ -induced tolerance.

In conclusion, we have shown in this study that Smad-dependent TGF β signaling is a crucial early step in the induction of TGF β 2-M ϕ -mediated tolerance. This event leads to changes in gene expression in TGF β 2-M ϕ , including the upregulation of Fc γ RI that we have shown here is required for tolerance induction in this system. As described above, there are a number of potential mechanisms by which Fc γ RI could be involved in TGF β 2-M ϕ -induced tolerance: ligation of Fc γ RI could drive IL-10 production and/or mediate shunting of antigen into both the Class I and Class II pathways that is required for induction of CD4 and CD8 regulatory cells, respectively, and/or antibody-bound Fc γ RI could facilitate “hand-off” of antigen to tolerance-inducing B cells. A large number of previous studies have shown that TGF β 2-M ϕ -induced tolerance is a highly potent form of antigen-specific tolerance that can inhibit both the afferent and efferent arm of the T cell response in *in vivo* model systems. The downregulation of activated autoreactive T cells is a goal for the successful treatment of autoimmune/inflammatory diseases, and an effective means of achieving this objective is not yet available. An understanding of the complex mechanisms that are involved in TGF β 2-M ϕ -induced tolerance will provide an important foundation for the development of this

strategy into a therapy that can be used for the prevention and/or treatment of a variety of autoimmune/inflammatory diseases.

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Abbreviations

ACAID	anterior chamber-associated immune deviation
ANOVA	analysis of variance
APC	antigen presenting cells
CFA	complete Freund's adjuvant
DN	dominant negative
DTH	delayed type hypersensitivity
i.v	intravenous
LPS	lipopolysaccharide
Mϕ	macrophage
Mϕ59	macrophage hybridoma cell line
OVA	ovalbumin
PEC	peritoneal exudates cells
s.c	subcutaneous
TGFβ	transforming growth factor β
WT	wild-type

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Figure 1.

Flow cytometric analysis of thioglycolate-elicited peritoneal exudate cells (PEC). Mice were injected with thioglycolate i.p. and 4 days later, PEC were harvested and cultured overnight with TGFβ2 and soluble protein antigen (LPS-free OVA). After culture, adherent cells were harvested, labeled with anti-F4/80 and anti-I-A antibodies then analyzed by flow cytometry.

Figure 2.

TGFβ2 induces translocation of Smad2/3 from cytosol to nucleus in primary macrophages (PEC). Control Mφ (PBS-treated) and TGFβ2-Mφ (TGFβ-treated) were analyzed for the presence of Smad2/3 in cytosolic and nuclear extracts by western blot (A). The relative nuclear/cytosol Smad2/3 ratios are displayed as mean±SEM. Statistical differences were analyzed using a Student's t test. * $p < 0.02$ compared to the control (B). PECs were labeled with anti-Smad2/3 and Alexa488-anti-IgG and DRAQ5, then analyzed by confocal microscopy (C). (n=3)

Figure 3. TGFβ2 induces translocation of Smad2/3 from cytosol to nucleus in a murine macrophage hybridoma cell line (Mφ59). Control Mφ59 and TGFβ2-Mφ59 were analyzed for the presence of Smad2/3 in cytosolic and nuclear extracts by western blot (A). The relative nuclear/cytosol Smad2/3 ratios are displayed as mean±SEM. Statistical differences were analyzed using a Student's t test. * $p < 0.02$ compared to the control (B). Mφ59 cells were labeled with anti-Smad2/3 and Alexa488-anti-IgG and DRAQ5, then analyzed by confocal microscopy (C). (n=3)

Figure 4.

Smad3-dependent TGFβ signaling is required for TGFβ2-Mφ-induced tolerance *in vivo*. Mφ59 cells untransfected (TGFβ Control) or stably transfected with either Smad2-DN, Smad3-DN or Smad2 and Smad3 (Smad2/3) DN (dominant negative) constructs were treated with TGFβ and OVA overnight, then injected i.v. into naïve mice ($n=5$ mice/group). After seven days, mice were immunized with OVA and CFA, then ear-challenged 10–14 days later. Ear swelling was measured after 24 hrs. Statistical differences were analyzed using ANOVA and the Tukey-Kramer multiple comparisons test. * $p<0.05$ compared to the positive control.

Figure 5.

Fc γ RI expression by TGF β 2-M ϕ is required for tolerance induction. mRNA extracted from Control M ϕ or TGF β 2-M ϕ (primary cells) was reverse transcribed and subjected to quantitative real-time PCR using Fc γ RI primers (A). TGF β 2-M ϕ from either wild-type (WT) or Fc γ RI-deficient (Fc γ RI^{-/-}) mice were injected i.v. into naïve WT mice ($n=5$ mice/group). After seven days, mice were immunized with OVA and CFA, then ear-challenged 10–14 days later. Ear swelling was measured after 24 hrs (B). Statistical differences were analyzed using ANOVA and the Tukey-Kramer multiple comparisons test. * $p<0.05$ compared to positive control.

Table 1Differential gene expression in TGF β -M ϕ vs Control M ϕ .

Gene Symbol	Genbank Accession #	Gene	Mean Fold- Δ
		IMMUNE-RELATED	
		Upregulated	
Thbs1	M62470	THROMBOSPONDIN 1	3.5
C1qb	M22531	COMPLEMENT COMPONENT 1, Q SUBCOMPONENT, BETA POLYPEPTIDE	3.2
Fcgr1	X70980	FC RECEPTOR, IGG, HIGH AFFINITY I	2.7
Hp	M96827	HAPTOGLOBIN	2.7
C1qa	X58861	COMPLEMENT COMPONENT 1, Q SUBCOMPONENT, ALPHA POLYPEPTIDE	2.7
Itgb5	AF022110	INTEGRIN BETA-5	2.7
C1qc	X66295	COMPLEMENT COMPONENT 1, Q SUBCOMPONENT, C CHAIN	2.5
Hgf	X72307	HEPATOCTE GROWTH FACTOR	2.5
Il17ra	U31993	INTERLEUKIN 17 RECEPTOR A	2.4
Ltc4s	U27195	LEUKOTRIENE C4 SYNTHASE	2.3
		Downregulated	
Cd200	AF029215	CD200 ANTIGEN	-2.2
Il1rn	L32838	INTERLEUKIN 1 RECEPTOR ANTAGONIST	-2.4
Ptpn22	M90388	PROTEIN TYROSINE PHOSPHATASE, NON-RECEPTOR TYPE 22 (LYMPHOID)	-2.7
Clec4a2	AJ133533	C-TYPE LECTIN DOMAIN FAMILY 4, MEMBER A2	-2.8
		CHEMOTAXIS	
		Upregulated	
S100a8	M83218	S100 CALCIUM BINDING PROTEIN A8 (CALGRANULIN A)	3.3
		Downregulated	
Ccl6	M58004	CHEMOKINE (C-C MOTIF) LIGAND 6	-2.5
Cxcl1	J04596	CHEMOKINE (C-X-C MOTIF) LIGAND 1	-2.8
Enpp2	AW122933	ECTONUCLEOTIDE PYROPHOSPHATASE/PHOSPHODIESTERASE 2	-2.9
Cxcl2	X53798	CHEMOKINE (C-X-C MOTIF) LIGAND 2	-4.2
Cxcl5	U27267	CHEMOKINE (C-X-C MOTIF) LIGAND 5	-4.3
Cxcl13	AF030636	CHEMOKINE (C-X-C MOTIF) LIGAND 13	-4.4
Ccl9	U49513	CHEMOKINE (C-C MOTIF) LIGAND 9	-4.5

Thioglycolate-elicited peritoneal exudate cells from C57BL/6 mice were harvested and cultured overnight with PBS (control) or TGF β 2 (TGF β -M ϕ). Adherent cells (>95% F4/80⁺) were collected, and RNA isolated, reversed transcribed and analyzed by micro-array. Mean fold- Δ indicates the fold (2) difference in gene expression between TGF β -M ϕ and Control M ϕ .