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Activation of a TGF-β-Specific Multistep Gene Expression Program in Mature Macrophages Requires Glucocorticoid-Mediated Surface Expression of TGF-β Receptor II[1]

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Alternatively activated (M2) macrophages regulate steady state-, cancer-, and inflammation-related tissue remodeling. They are induced by Th2-cytokines and glucocorticoids (GC). The responsiveness of mature macrophages to TGF-β, a cytokine involved in inflammation, cancer, and atherosclerosis, is currently controversial. Recently, we demonstrated that IL-17 receptor B is up-regulated in human monocyte-derived macrophages differentiated in the presence of Th2 cytokines IL-4 and TGF-β1. In this study, we show that mature human macrophages differentiated in the presence of IL-4, and dexamethasone ($M2_{IL\text{-}4/GC}$) but not $M2_{IL\text{-}4}$ responds to TGF-β1 which induced a gene expression program comprising 111 genes including transcriptional/signaling regulators (*ID3* and *RGS1*), immune modulators (*ALOX5AP* and *IL-17 receptor B*) and atherosclerosis-related genes (*ALOX5AP*, *ORL1*, *APOC1*, *APOC2*, and *APOE*). Analysis of molecular mechanism underlying GC/TGF-β cooperation revealed that surface expression of TGF-βRII was high in $M2_{GC}$ and $M2_{IL\text{-}4/GC}$, but absent from $M2_{IL\text{-}4}$, whereas the expression of TGF-βRI/II mRNA, TGF-βRII total protein, and surface expression of TGF-βRIII were unchanged. GC dexamethasone was essential for increased surface expression of functional TGF-βRII because its effect was observed also in combination with IL-13, M-CSF, and GM-CSF. Prolonged Smad2-mediated signaling observed in TGF-β1-treated $M2_{IL\text{-}4/GC}$ was due to insufficient activity of negative feedback mechanism what can be explained by up-regulation of SIRT1, a negative regulator of Smad7, and the retention of TGF-βRII complex on the cell surface. In summary, mature human M2 macrophages made permissive to TGF-β by GC-induced surface expression of TGF-βRII activate in response to TGF-β1, a multistep gene expression program featuring traits of macrophages found within an atherosclerotic lesion. *The Journal of Immunology,* **2008, 180: 6553–6565.**

Macrophages are key regulators of homeostatic and pathologic processes in all tissues of an organism. During development under physiological conditions, macrophages encounter tissue specific signals via cell-cell contacts, soluble factors, and interactions with the extracellular matrix. Upon inflammation, monocytes migrating into tissue sites from which danger signals originate differentiate under the predominant influence of proinflammatory mediators. Termination of inflammatory reactions, wound healing, and tissue remodeling are conducted by re-differentiated and newly developed anti-inflammatory macrophages. These two major functional states, i.e., response to danger and tissue remodeling, were translated into a concept of classical and alternative macrophage activation (1–3). Alternative activation (M2) was primarily described as a response of macrophages to Th2 cytokines, such as IL-4, IL-13, and IL-10, as well as to anti-inflammatory mediators, such as glucocorticoids (GCs)[4] (4). The diversity of signals necessary for the development of M2 prompted a deeper analysis of this macrophage population (5–7). It has been shown, for example, that stimulation of macrophages by the GC dexamethasone leads to up-regulation of endocytic receptors such as CD163(8) and stabilin-1 (9–12), and to an increase of their phagocytic and endocytic potential (4). In contrast to dexamethasone, IL-4 induced the expression of extracellular matrix components and matrix remodeling enzymes (4, 13).

The role of TGF-β, another pleiotropic anti-inflammatory cytokine, in macrophage biology is less well defined. Previously, we have shown that stimulation of macrophages by the combination of IL-4 and TGF-β1 leads to an increased expression of IL-17 receptor B (IL17RB), the receptor for Th2-associated cytokine IL17E/IL-25(14). In disease IL-4 and TGF-β both play an important role in the development of atherosclerotic lesions by stimulating the development of a specific phenotype of macrophages (15–17). Tumor cells producing high levels of IL-4 and TGF-β1 condition tumor infiltrating monocytes/macrophages to support tumor growth and immune escape (18, 19).

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[4] Abbreviations used in this paper: GC, glucocorticoid; IL17RB, IL-17 receptor B; R-Smad, receptor-regulated Smad; FC, fold change.

TGF-β acts via a well-defined signaling cascade activated by engagement of cell surface serine/threonine protein kinases known as type I and type II receptors. TGF-β1 binds to a heteromeric complex composed of TGF-βRII and TGF-βRI. After ligand binding, TGF-βRII phosphorylates and activates TGF-βRI (20). Activated TGF-βRI transmits its signal into the cell via phosphorylation of the receptor-regulated Smads (R-Smads), Smad2 and Smad3. Activated R-Smads form heteromeric complexes with Smad4 and translocate to the nucleus. In the nucleus, Smads bind to Smad binding elements and regulate the transcription of target genes in a cell-specific manner. The ability of cells to respond to cytokines of the TGF-β family is usually regulated by the spectrum of receptors expressed and the level of their expression. It was reported that circulating monocytes express ~400 functional TGF-β receptor complexes per cell (21); it is, however, widely accepted that during macrophage differentiation and activation receptor numbers are dramatically reduced, making mature macrophages refractory to TGF-β stimulation (22).

In this study, we investigated whether and to what extent mature macrophages are able to respond to TGF-β. We established that mature $M2_{IL\text{-}4/dex}$, but not mature $M2_{IL\text{-}4}$, readily respond to TGF-β1 by induction of a specific, multistep gene expression program. GCs rendered macrophages permissive for TGF-β by maintaining surface expression of TGF-βRII. This effect of GC was found not only in combination with IL-4 but also in combination with IL-13, M-CSF, and GM-CSF indicating that GC is a key factor responsible for the increased surface exposure of TGF-βRII. Time course experiments showed that the presence of GCs is required during the differentiation of monocytes to macrophages for maintaining surface expression of TGF-βRII. Dose response experiments showed that physiological concentrations of GCs are sufficient to secure a baseline TGF-β response in macrophages that may be amplified upon therapeutic use of GCs. The amount of TGF-βRII translocated to the cell surface under dexamethasone treatment was high enough to secure prolonged TGF-β1-mediated signaling. Reduced activity of negative feedback mechanisms, due to GC-mediated induction of SIRT1, a negative Smad 7 regulator, may also contribute to this effect. In $M2_{IL\text{-}4/dex}$, the TGF-β1-activated gene expression program comprises "early response" genes involved in transcriptional regulation and signaling that may mediate secondary TGF-β effects, and "late response" genes involved in (Th2) immune modulation and lipid metabolism/atherosclerosis. We suggest that TGF-β-stimulated $M2_{IL\text{-}4/dex}$ have a dual physiological role as regulators of inflammatory reactions and as major players in lipid uptake and processing in health and disease.

Materials and Methods

Cells and mediators

The isolation and cultivation of human monocytes/macrophages was done as described (4, 6). Briefly: the cells were purified from individual buffy coats using density gradients followed by $CD14^+$ magnetic cell sorting (Miltenyi Biotech). Macrophages were cultured at 1×10^6 cell/ml in X-vivo 10 serum free medium (Cambrex), and supplemented with cytokines and/or dexamethasone as indicated, for 5 days. A detailed protocol is available at the http://www.methods.info/index.html website.

Human IL 4, IL-13, M-CSF, GM-CSF, and TGF-β1 were from PeproTech. Cytokines were used at a final concentration of 10 ng/ml. Dexamethasone (Sigma-Aldrich) was used at 1×10^{-7} M or as indicated.

Microarray analysis

For oligonucleotide microarray analysis, total RNA was isolated out of macrophages cultivated in the presence of IL-4 or IL-4 in combination with dexamethasone for 5 days and then additionally stimulated by TGF-β1 for 3 or 24 h. Labeling, hybridization, and scanning of human genome U133 plus 2.0 Genechip arrays were conducted by the Medical Research Center. Five independent donors were analyzed for each stimulation. The data were submitted to Gene Expression Omnibus database, accession number of the study is GSE7568. Raw data from Affymetrix CEL files were analyzed using SAS software package Microarray Solution version 1.3 (SAS Institute). Custom Chip Definition File (23), which has a updated probe set definition, was applied to map the probes to gene. Gene annotation was obtained through the Affymetrix NetAffx website (http://www.affymetrix.com/analysis/index.affx). Quality control, normalization, and statistical modeling were performed by array group correlation, mixed model normalization, and mixed model analysis respectively. Analysis of differential gene expression was based on a loglinear mixed model of perfect matches (24). A false discovery rate of $a = 0.05$ with Bonferroni-correction for multiple testing was used to set the level of significance.

FACS analysis

For FACS analysis, 5×10^5 cells were washed twice with PBS and resuspended in 100 μl of 1% BSA solution in PBS, containing Ab diluted at 1/100 or a corresponding isotype control. After 1-h incubation on ice, the cells were washed three times with 1% BSA in PBS and re-suspended in 300 μl of the same buffer. Stained cells were analyzed using FACS Calibur (BD Biosciences). Obtained results were evaluated with WinMDI software. Statistical analysis of the flow cytometry data was performed using statistical functions of the Microsoft Excel 2002 professional program.

RNA isolation and cDNA synthesis

RNA isolation was performed using RNeasy Mini kit (Qiagen). For the first strand cDNA synthesis, 500 ng of total RNA was treated with 2 U RNase free DNase (Fermentas). DNase treated RNA was used for reverse transcription with Superscript III reverse transcriptase (Invitrogen) using oligo(dT) primers.

Analysis of TGF-βRII protein expression

Samples for Western blotting were prepared by direct lysis of macrophages in Laemmli loading buffer supplemented with 2-ME. Protein loading was controlled by parallel gel staining with GelCode Blue Stain reagent (Pierce) and by GAPDH detection using anti-GAPDH rabbit polyclonal Ab (Abcam; Catalog no. Ab9485-100). TGF-βRII was detected using anti-TGF-βRII rabbit polyclonal Ab (Santa Cruz Biotechnology; Catalog no. Sc-1700). Pierce Super Signal Pico system was used for signal detection.

Real-time RT-PCR analysis

Relative quantification of gene expression was performed using real-time RT-PCR analysis. The following predeveloped TaqMan assays (Applied Biosystems) were used: Hs00178696_m1 for Smad7, Hs00171409_m1 for ID3, Hs00610319_m1 for ALK5, Hs00234253_m1 for TGF-βRII, Hs00224203_m1 for Smurf2, Hs00180524_m1 for SKIL, Hs01009006_m1 for SIRT1, Hs00214108_m1 for RNF111, and Hs00356621_g1 for FKBP1A. The assays for IL17RB, OLR1, and the house keeping gene GAPD were designed in our laboratory (Table I). All reactions were performed using TaqMan Universal PCR master mix on Applied Biosystems 7000 Real-Time PCR system (Applied Biosystems). The expression levels of analyzed genes were normalized to GAPD mRNA expression. Statistical analysis of the real-time RT-PCR data was performed using statistical functions of the Microsoft Excel 2002 professional program.

Protein phosphorylation analysis

For the analysis of protein phosphorylation, $1–3 \times 10^6$ cells were lysed in 50 μl of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% NP40, 5 mM NaF, 0.25% Na deoxycholate, 2 mM NaVO3, and 1× Complete protein inhibitors (Roche). The samples were then separated in a 12% PAGE and transferred to a nitrocellulose membrane. For detection, rabbit anti-human Smad2 mAb (Cell Signaling Technology; Catalog no. 3102) was used at a 1/1000 dilution and rabbit anti-human phospho-Smad2 mAb (Cell Signaling Technology; no. 3108) at a 1/500 dilution. As a secondary Ab, anti-rabbit IgG HRP-linked whole Ab (GE Healthcare) was used at a dilution of 1/5000. Chemoluminescence detection was performed using SuperSignal Pico peroxidase substrate (Pierce).

Results

TGF-β1 up-regulates the expression of IL17RB not only in monocytes, but also in mature M2 macrophages

We previously showed a significant induction of IL17RB expression in monocytes when differentiated into macrophages in the

Table I. *Oligonucleotides used for real-time RT-PCR analysis*

Gene	Primer Name	Primer Sequence	Function
GAPD	F848	5′-CATCCATGACAACTTTGGTATCGT	Forward
	R848	5′-CAGTCTTCTGGGTGGCAGTGA	Reverse
	P849	FAM-AAGGACTCATGACCACAGTCCATGCC-BHQ1	Probe
IL17RB	F846	5′-AACACAGCACTATCATCGGGTTT	Forward
	R846	5′-CCACTGAAGCTCGCGTTTG	Reverse
	P847	FAM-CAGGTGTTTGAGCCACACCAGAAGA-BHQ1	Probe
OLR1	F943	5′-TTGGATGCCAAGTTGCTGAA	Forward
	R943	5′-GAATGGAAAACTGGAATAGGAAATTG	Reverse
	P943	FAM-AGCACAGCTGATCTGGACTTCATCCAG-BHQ1	Probe

continuous presence of both IL-4 and TGF-β1 starting on day 0 of cell culture. In this experimental setting, IL-4 alone caused only weak expression of IL17RB, whereas TGF-β1 alone did not induce any detectable IL17RB expression (14). In this study, we tested the ability of TGF-β1 to induce IL17RB in mature alternatively activated macrophages (day 5 of cell culture), because the ability of mature macrophages to respond to TGF-β is a controversial issue (21, 22). As TGF-β signaling is a relatively rapid process (25), we tested whether the expression of IL17RB can be up-regulated by TGF-β1 within a short time period. Macrophages were differentiated in the presence of IL-4 ($M2_{IL\text{-}4}$) or IL-4 in combination with dexamethasone ($M2_{IL\text{-}4/dex}$) for 5 days, at the end of which, TGF-β1 was added to the culture medium. Cells were harvested 3 and 24 h after the addition of TGF-β1, and the expression of IL17RB mRNA was analyzed using real-time RT-PCR (Fig. 1*A*). No induction of IL17RB mRNA expression was observed after 3 h of stimulation by TGF-β1 in either macrophage population. After 24 h of stimulation by TGF-β1, we observed a statistically significant 5-fold up-regulation of the expression of IL17RB mRNA in $M2_{IL\text{-}4/dex}$, whereas the observed 2-fold induction of IL17RB mRNA expression in $M2_{IL\text{-}4}$ did not prove statistically significant. These findings indicate that differentiated M2 retain the ability to respond to TGF-β1 stimulation and that this response is significantly stronger in $M2_{IL\text{-}4/dex}$ compared with $M2_{IL\text{-}4}$.

Expression profiling of TGF-β-stimulated M2 macrophages

Because mature $M2_{IL\text{-}4}$ and $M2_{IL\text{-}4/dex}$ differed with respect to IL17RB induction by TGF-β1, we asked whether other TGF-β-inducible genes were also differentially regulated in these macrophages. In search of the TGF-β-inducible synexpression group of genes in differentiated M2 macrophages, we performed expression

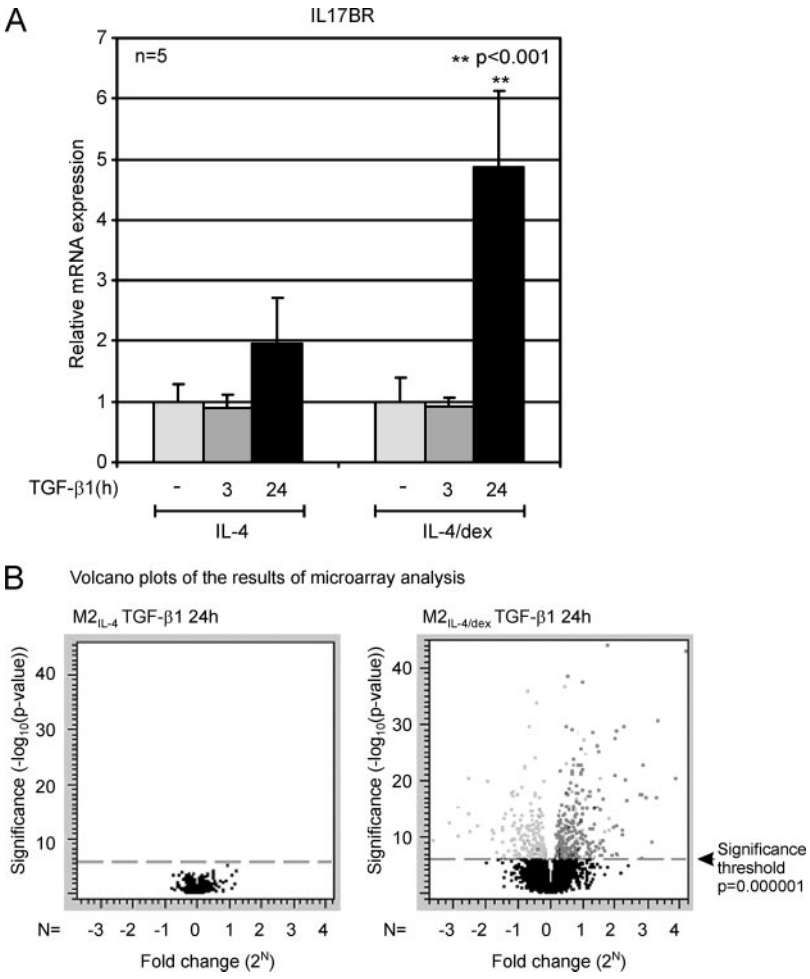

FIGURE 1. Analysis of the response of mature macrophages to TGF-β1. *A*, Macrophages were first stimulated for 5 days by IL-4 or IL-4/dex, and subsequently by TGF-β1 as indicated. The expression of IL17RB mRNA was measured by real-time RT-PCR and normalized to the expression of GAPD. IL17RB mRNA expression in samples without TGF-β1 stimulation was taken as 1. *B*, Microarray analysis of $M2_{IL\text{-}4}$ and $M2_{IL\text{-}4/dex}$ activated by TGF-β1. Macrophages were differentiated for 5 days as indicated and then stimulated by TGF-β1. Volcano plots of genes found to be overexpressed after 24 h of TGF-β1 stimulation are presented.

Table II. *Functional groups of genes up-regulated by TGF-β 1 with FC ≥ 2*

AffyID	Gene_Title	FC 3 h vs Control	FC 24 h vs Control	FC 24 h vs 3 h
Transcription				
207826_s_at	*ID3*: inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	10.0	6.9	0.7
214445_at	*ELL2*: elongation factor, RNA polymerase II, 2	2.5	2.6	1.0
204253_s_at	*VDR*: vitamin D (1,25-dihydroxyvitamin D3) receptor	2.5	1.8	0.7
206472_s_at	*TLE3*: transducin-like enhancer of split 3	2.3	1.8	0.8
206127_at	*ELK3*: ETS-domain protein (SRF accessory protein 2)	2.3	1.6	0.7
219433_at	*BCOR*: BCL6 corepressor	2.2	1.2	0.5
204197_s_at	*RUNX3*: runt-related transcription factor 3	2.2	1.0	0.5
208328_s_at	*MEF2A*: MADS box transcription enhancer factor 2, polypeptide A (myocyte enhancer factor 2A)	2.0	1.5	0.7
201473_at	*JUNB*: jun B proto-oncogene	2.0	1.5	0.7
209579_s_at	*MBD4*: methyl-CpG binding domain protein 4	1.7	2.2	1.3
1569108_a_at	*ZNF589*: zinc finger protein 589	1.4	2.1	1.5
209189_at	*FOS*: v-fos FBJ murine osteosarcoma viral oncogene homolog	1.3	2.7	2.2
204959_at	*MNDA*: myeloid cell nuclear differentiation antigen	1.2	2.5	2.1
TGF-β and BMP signaling				
201185_at	*PRSS11*: protease, serine, 11 (IGF binding)	1.3	10.1	7.7
204790_at	*SMAD7*: SMAD, mothers against DPP homolog 7 (*Drosophila*)	6.2	5.1	0.8
204948_s_at	*FST*: follistatin	1.2	2.7	2.2
207069_s_at	*SMAD6*: SMAD, mothers against DPP homolog 6 (*Drosophila*)	2.2	1.8	0.8
205596_s_at	*SMURF2*: SMAD specific E3 ubiquitin protein ligase 2	2.6	1.5	0.6
Immune response				
220491_at	*HAMP*: hepcidin antimicrobial peptide	6.0	14.6	2.4
204174_at	*ALOX5AP*: arachidonate 5-lipoxygenase-activating protein	1.1	8.7	7.7
202988_s_at	*RGS1*: regulator of G-protein signaling 1	8.6	7.0	0.8
219434_at	*TREM1*: triggering receptor expressed on myeloid cells 1	1.5	4.1	2.7
219255_x_at	*IL17RB*: IL 17 receptor B	1.1	3.8	3.6
206618_at	*IL18R1*: IL 18 receptor 1	1.0	2.8	2.6
1555116_s_at	*SLC11A1*: solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	1.3	2.7	2.0
208771_s_at	*LTA4H*: leukotriene A4 hydrolase	1.2	2.5	2.1
203788_s_at	*SEMA3C*: sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	1.3	2.3	1.7
208071_s_at	*LAIR1*: leukocyte-associated Ig-like receptor 1	1.8	2.2	1.2
209201_x_at	*CXCR4*: chemokine (C-X-C motif) receptor 4	2.4	1.8	0.7
Lipid binding, transport, processing				
210004_at	*OLR1*: oxidized low density lipoprotein (lectin-like) receptor 1	6.0	20.0	3.3
204561_x_at	*APOC2*: apolipoprotein C-II	1.7	7.7	4.5
204416_x_at	*APOC1*: apolipoprotein C-I	1.4	3.1	2.2
203509_at	*SORL1*: sortilin-related receptor, L(DLR class) A repeats-containing	1.4	2.6	1.8
203381_s_at	*APOE*: apolipoprotein E	1.3	2.5	2.0
201186_at	*LRPAP1*: low density lipoprotein receptor-related protein associated protein 1	1.2	2.4	2.0
1570432_at	*ABCG1*: ATP-binding cassette, sub-family G (WHITE), member 1	1.9	2.0	1.0
Cell adhesion				
1552806_a_at	*SIGLEC10*: sialic acid binding Ig-like lectin 10	1.7	4.8	2.9
1559921_at	*PECAM1*: platelet/endothelial cell adhesion molecule (CD31 Ag)	1.1	3.1	2.8
202351_at	*ITGAV*: integrin, αV (vitronectin receptor, α polypeptide, Ag CD51)	3.2	3.0	0.9
201042_at	*TGM2*: transglutaminase 2 (C polypeptide, protein-glutamine-γ-glutamyltransferase)	1.1	2.7	2.4
201389_at	*ITGA5*: integrin, α5 (fibronectin receptor, α polypeptide)	2.5	2.0	0.8
205204_at	*NMB*: neuromedin B	1.1	2.0	1.8

profiling using oligonucleotide microarrays. Primary monocytes isolated from buffy coats were cultivated for 5 days in the presence of IL-4 or IL-4 in combination with dexamethasone (five independent donors for each stimulation). These mature macrophages were then stimulated by TGF-β1 for 24 h. RNA from stimulated macrophages and nonstimulated controls was isolated and used for the hybridization of microarrays. Analysis of the expression profiles, using a highly stringent setting was unable to detect a statistically significant change of gene expression in $M2_{IL\text{-}4}$ in response to TGF-β1 (Fig. 1*B*). This result was in accordance with our finding regarding IL17RB (Fig. 1*A*) and with other reports indicating that mature macrophages do not respond to TGF-β efficiently (22). In $M2_{IL\text{-}4/dex}$, however, TGF-β1 up-regulated the expression of 90 genes with a fold change (FC) ≥ 2 in a statistically significant manner ($p < 0.000001$). Based on these findings, we limited further gene expression profiling analysis to $M2_{IL\text{-}4/dex}$. To investigate the kinetics of gene expression upon stimulation of M2 macrophages by TGF-β1, we performed an additional microarray analysis of $M2_{IL\text{-}4/dex}$ stimulated by TGF-β1 for 3 h. Analysis of gene expression profiles in $M2_{IL\text{-}4/dex}$ stimulated by TGF-β1 for 3 and 24 h revealed two groups of genes with different kinetics of expression change. The "early response" group comprises 44

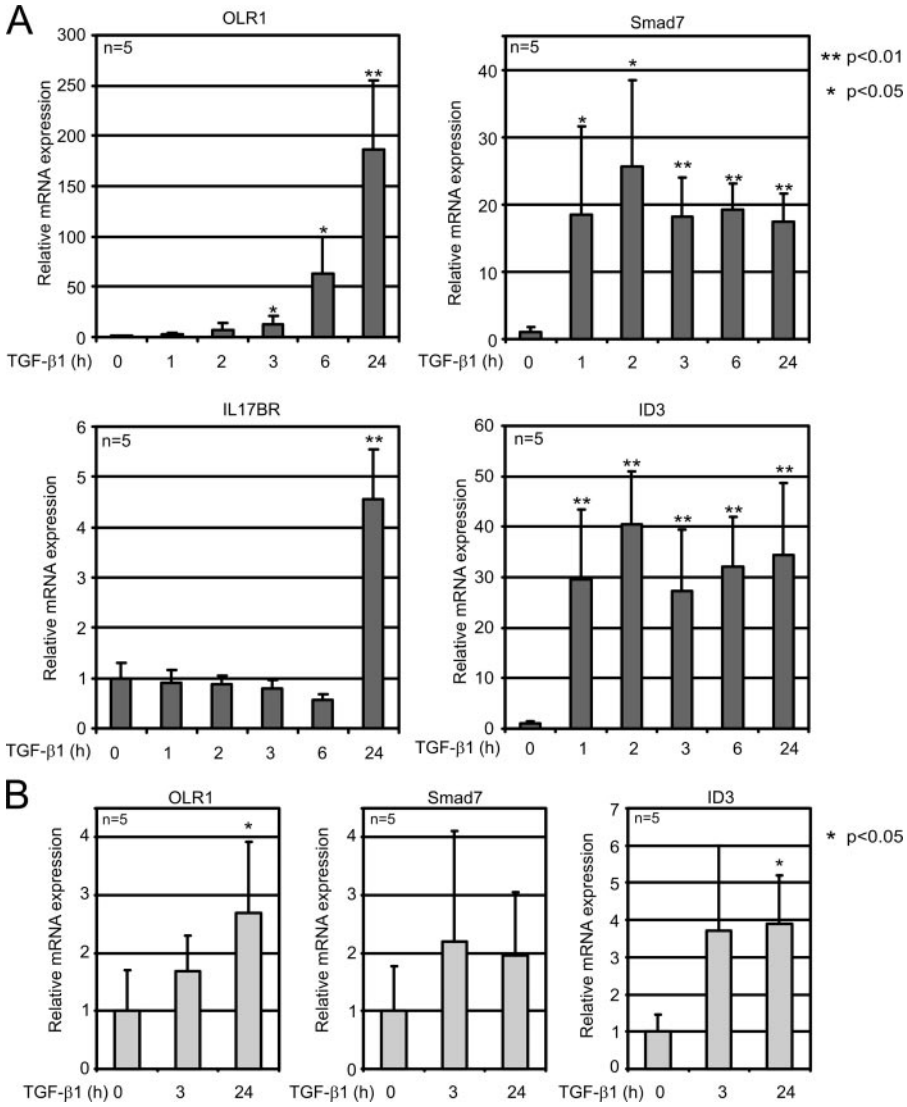

FIGURE 2. Real-time RT-PCR analysis of the expression of OLR1, Smad7, IL17RB, and ID3 in $M2_{IL\text{-}4/dex}$ and $M2_{IL\text{-}4}$. Macrophages were differentiated for 5 days either in the presence of IL-4 in combination with dexamethasone (*A*) or IL-4 alone (*B*), and then stimulated by TGF-β1 as indicated. Expression levels were normalized to the expression levels of GAPD mRNA. *p* values were determined using paired, two-sided *t* test.

genes whose expression showed a statistically significant increase (FC $\geq$ 2) already after 3 h of stimulation. The "late response" group comprises 90 genes that showed a FC $\geq$ 2 increase after 24 h of stimulation. The overlap of both groups comprises 23 genes with a FC $\geq$ 2 at both time points. These expression profiles indicate that TGF-β1 induces a complex gene expression program in $M2_{IL\text{-}4/dex}$ that is tightly regulated kinetically. The presence of early and late TGF-β1 response genes may be an indication of the secondary activation of other signaling pathways beyond the primary TGF-β1 signaling cascade (26).

Within the set of genes up-regulated by TGF-β1 with a FC $\geq$ 2 after 3 h, 24 h, or both, we identified five functional groups (Table II). The first group comprises 12 genes involved in transcriptional regulation, which were mainly (9 out of 12) "early response" genes. Among them were known Smad3-dependent targets of TGF-β signaling, i.e., *JUNB* and *FOS* (27) (Table II). The second group is comprised of the five genes involved in the regulation of TGF-β and bone morphogenetic protein signaling, including the inhibitory Smads *Smad7* and *Smad6* (28). Three out of five genes of this group belong to the "early response" category (Table II). The other three groups, i.e., immune response modulation, lipid transport and processing, and cell adhesion, are mainly comprised of "late response" genes (24 out of 25). The genes involved in immune response modulation include various receptors, soluble factors, signaling molecules, and enzymes involved in leukotriene synthesis; the genes involved in lipid binding, transport, and processing include the TGF-β inducible receptor for oxidized low density lipoprotein, *OLR1*, (29), as well as several apolipoproteins (Table II). This classification of the up-regulated genes indicates that stimulation of M2 macrophages by TGF-β1 induces the expression of various transcriptional regulators that in turn contribute to the development of a macrophage phenotype with an enhanced capacity for lipid uptake and processing and with specific immuno-modulatory properties.

Confirmation of the expression profiling data

To confirm the data obtained in the microarray experiments, we selected *Smad7* and *ID3* as early response genes, *OLR1* as an early-to-late gene, and *IL17RB* as a late response gene. $M2_{IL\text{-}4/dex}$ were stimulated by TGF-β1 on day 5 of culture, and cells were harvested after 1, 2, 3, 6, and 24 h. Real-time RT-PCR analysis revealed that OLR1 expression increased continuously throughout the analyzed time period (Fig. 2*A*). The up-regulation of OLR1 expression became statistically significant after 3 h of stimulation. In contrast to OLR1, the expression of Smad7 and ID3 mRNAs increased significantly already after 1 h of TGF-β1 stimulation and remained at this level during the

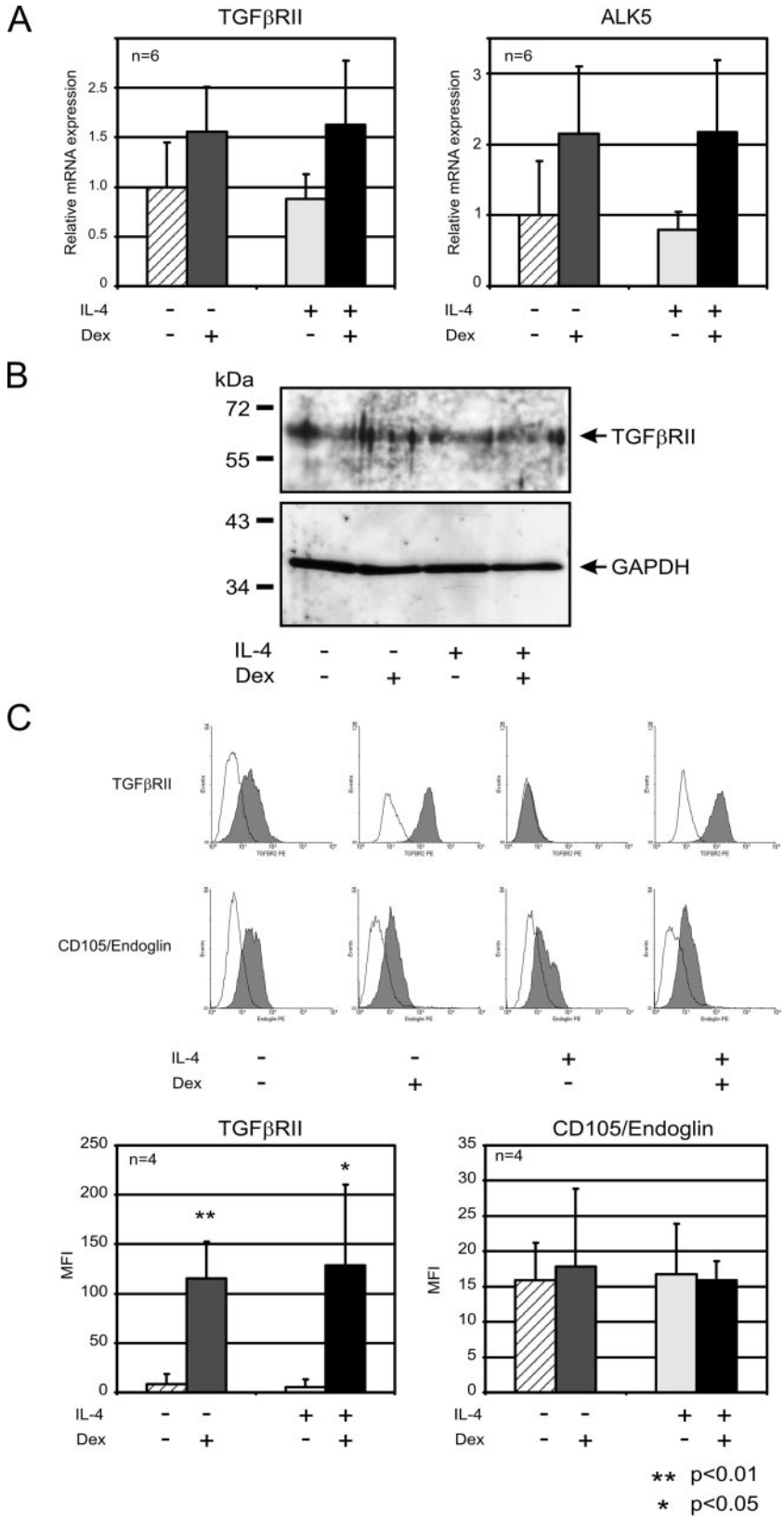

FIGURE 3. Analysis of the TGF-β receptor system in M2 macrophages. Macrophages were differentiated in the presence of IL-4 and dexamethasone as indicated. *A*, The mRNA expression of TGF-βRII and TGF-βRI (ALK5) was analyzed using real-time RT-PCR. Expression levels were normalized to the expression levels of GAPD mRNA. *B*, Western blot analysis of TGF-βRII expression. Western blot with GAPDH was used to show equal amount of protein lysate loaded. *C*, Surface expression of TGF-βRII and TGF-βRIII (endoglin) was analyzed using flow cytometry. *p* values were determined using paired, two-sided *t* test.

24 h of continued stimulation (Fig. 2*A*). In accordance with previously obtained data (Fig. 1*A*, Table II), a statistically significant increase of the expression of IL17RB mRNA was observed only after 24 h of stimulation (Fig. 2*A*). Notably, the values for the up-regulation of the genes obtained with real-time RT-PCR experiments were higher than the corresponding values obtained by analysis of microarray data. For example, the expression of *OLR1* increased 19.7-fold according to the microarray analysis, but 180-fold according to real-time RT-PCR. This difference in sensitivity between the microarray and real-time RT-PCR data prompted us to test whether a statistically significant up-regulation of the TGF-β1 early response

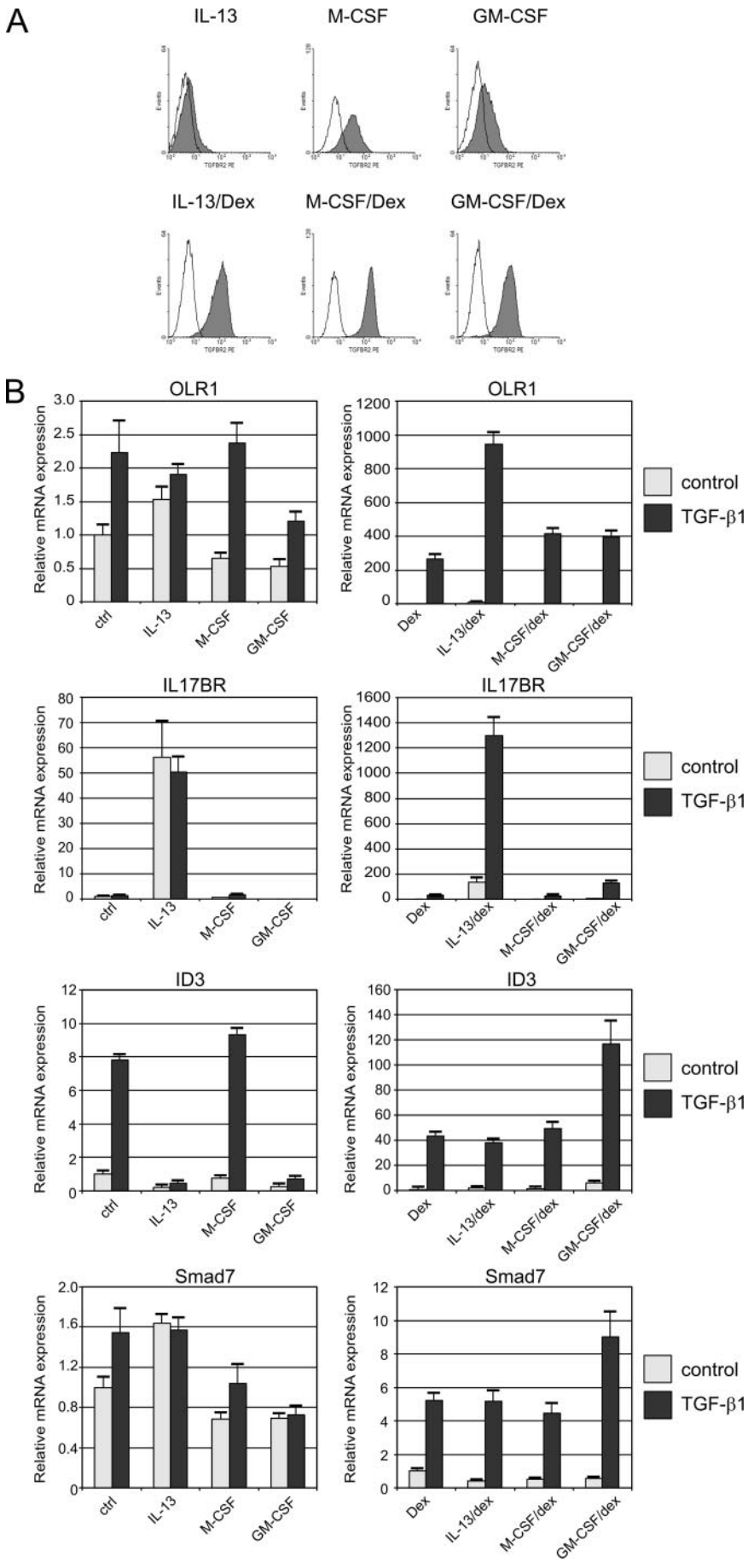

FIGURE 4. Analysis of TGF-βRII cell surface expression and of TGF-β-dependent target genes in macrophages stimulated by either IL-13, M-CSF, and GM-CSF alone or in combination with dexamethasone. *A*, Macrophages were stimulated as indicated for 5 days, and surface expression of TGF-βRII was analyzed using flow cytometry. *B*, Macrophages were stimulated as indicated for 5 days and then stimulated by TGF-β1 for 24 h. Analysis of relative mRNA expression of indicated genes was performed using real-time RT-PCR.

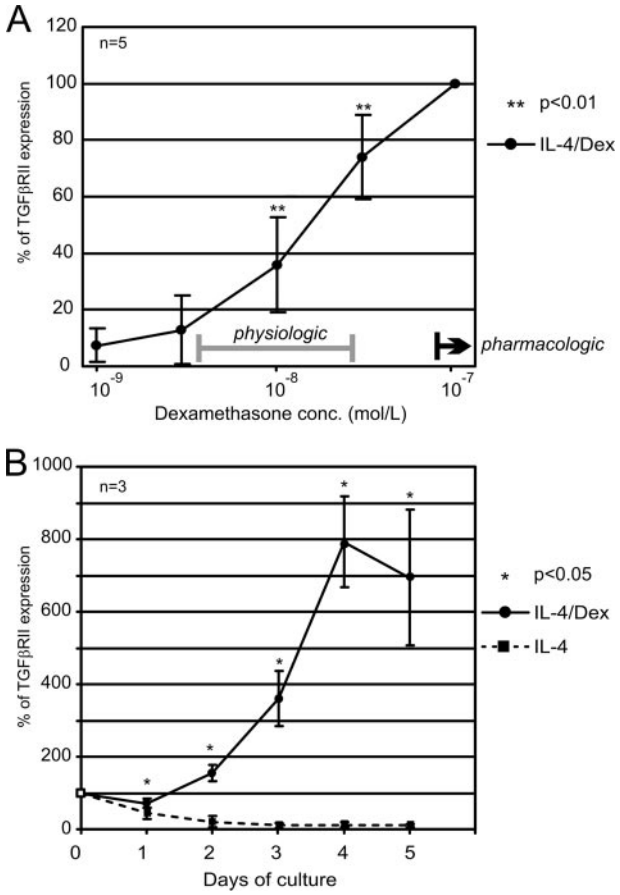

FIGURE 5. Analysis of dose response and kinetics of the surface expression of TGF-βRII in macrophages. *A*, Macrophages were differentiated for 5 days in the presence of IL-4 and different dexamethasone concentrations. Surface expression of TGF-βRII was measured using flow cytometry. Expression value obtained at 1×10^{-7} M dexamethasone was taken as 100%. Gray bar indicates a physiologic range of GC concentrations; black arrow indicates the beginning of pharmacologic concentration range. *B*, Macrophages were stimulated by IL-4 alone (dotted line) or IL-4 in combination with dexamethasone (solid line). Surface expression of TGF-βRII was measured by flow cytometry at the time points indicated. TGF-βRII expression value obtained on freshly isolated monocytes was taken as 100%. *p* values were determined using paired, two-sided *t* test.

genes could also be observed in $M2_{IL-4}$ using real-time RT-PCR.

Analysis of "immediate response" genes in $M2_{IL-4}$ stimulated by TGF-β1

We performed a real-time RT-PCR analysis of OLR1, Smad7, and ID3 mRNA expression in $M2_{IL-4}$ stimulated by TGF-β1 for 3 and 24 h at day 5 of culture (Fig. 2*B*). We were able to show a statistically significant up-regulation of ID3 and OLR1 mRNA expression after 24 h of stimulation. The up-regulation of the genes in $M2_{IL-4}$, however, was far less pronounced than in $M2_{IL-4/dex}$. The expression of Smad7 was up-regulated 2-fold in $M2_{IL-4}$ (not significant) and 20-fold in $M2_{IL-4/dex}$. A similar difference was observed for ID3 (4-fold in $M2_{IL-4}$ and 30-fold in $M2_{IL-4/dex}$) and OLR1 (1.6-fold in $M2_{IL-4}$ and 12-fold in $M2_{IL-4/dex}$) after 3 h of stimulation (Fig. 2). After 24-h stimulation, this difference became even stronger for OLR1 (2.5-fold in $M2_{IL-4}$ and 180-fold in $M2_{IL-4/dex}$) (Fig. 3). Taken together with the microarray data (Fig. 1*B*), our results indicate that dexamethasone has a global effect on the TGF-β-dependent gene expression program in M2 macrophages rather than solely modulating the expression of single genes, such as IL17RB or others (14, 30). We hypothesized that $M2_{IL-4}$ and $M2_{IL-4/dex}$ might show fundamental molecular differences in TGF-β signaling.

Dexamethasone regulates surface expression of TGF-βRII

Because differential regulation of TGF-β receptors on the mRNA level has been described in macrophages (22, 31) and tumor cell lines (32), we tested whether the differential response of different populations of M2 macrophages to TGF-β might be explained by differences in the expression of TGF-βRI (ALK5) and TGF-βRII in mature $M2_{IL-4}$ and $M2_{IL-4/dex}$. For this analysis, primary human monocytes were cultivated for 5 days in medium alone or in medium supplemented with dexamethasone, IL-4, or a combination of both. Using real-time RT-PCR analysis, we could show that dexamethasone induced an ~2-fold increase of both TGF-βRII and ALK5 mRNA; however, these increases were not statistically significant (Fig. 3*A*) and these small differences in TGF-βRII and ALK5 expression could not explain the dramatic differences in the effects of TGF-β on $M2_{IL-4}$ and $M2_{IL-4/dex}$. In addition, we investigated whether dexamethasone induces more efficient translation of TGF-βRII mRNA leading to higher amounts of TGF-βRII protein. Western blot analysis with total cell lysates did not reveal any significant differences in the level of TGF-βRII protein in macrophages cultivated in the presence or absence of dexamethasone (Fig. 3*B*).

As subcellular localization of the receptors rather than total protein content might influence TGF-β responsiveness of macrophages, we next investigated the surface expression of TGF-βRII and endoglin (an accessory receptor for TGF-β) using flow cytometry. We found that TGF-βRII was highly expressed on the surface of $M2_{dex}$ and $M2_{IL-4/dex}$, whereas it was not detectable on the surface of $M2_{IL-4}$ (Fig. 3*C*). As expected, TGF-βRII was also detected on the surface of freshly isolated monocytes (data not shown), whereas mature macrophages cultivated in medium alone (control macrophages) – similar to $M2_{IL-4}$– showed little or no TGF-βRII surface expression (Fig. 3*C*). Statistical analysis of the effect of dexamethasone on the surface expression of TGF-βRII showed a 13-fold higher TGF-βRII expression in $M2_{Dex}$ in comparison with control cells and more than a 20-fold difference between $M2_{IL-4/dex}$ and $M2_{IL-4}$ (Fig. 3*C*). In contrast, the surface expression of endoglin, an accessory receptor for TGF-β, was expressed on a similar level in all macrophage populations analyzed (Fig. 3*C*) with no statistically significant differences observed (Fig. 3*C*).

Dexamethasone-stimulated macrophages are permissive for TGF-β activation independently of additional stimulation

To test whether dexamethasone increases surface expression of TGF-βRII in the presence of cytokines other than IL-4, we cultivated macrophages in the presence of IL-13, M-CSF, and GM-CSF with or without addition of dexamethasone. Analysis of the surface expression of TGF-βRII after 5 days of culture revealed that IL-13 caused reduction of TGF-βRII expression to nondetectable levels similar to IL-4 (Fig. 4*A*). Stimulation by M-CSF preserved TGF-βRII expression on the cell surface of mature macrophages to some extent, whereas GM-CSF preserved detectable, but quite weak, TGF-βRII expression (Fig. 4*A*). Combination of dexamethasone with any of these cytokines dramatically increased the amount of TGF-βRII on the cell surface (Fig. 4*A*).

To study the response of these macrophage populations to TGF-β1, the mRNA expression of OLR1, IL17RB, ID3, and Smad7 was analyzed. Macrophages were stimulated with either IL-13, M-CSF, or GM-CSF with or without addition of dexamethasone for 5 days, after which time TGF-β1 was added for 24 h (Fig. 4*B*). Real-time

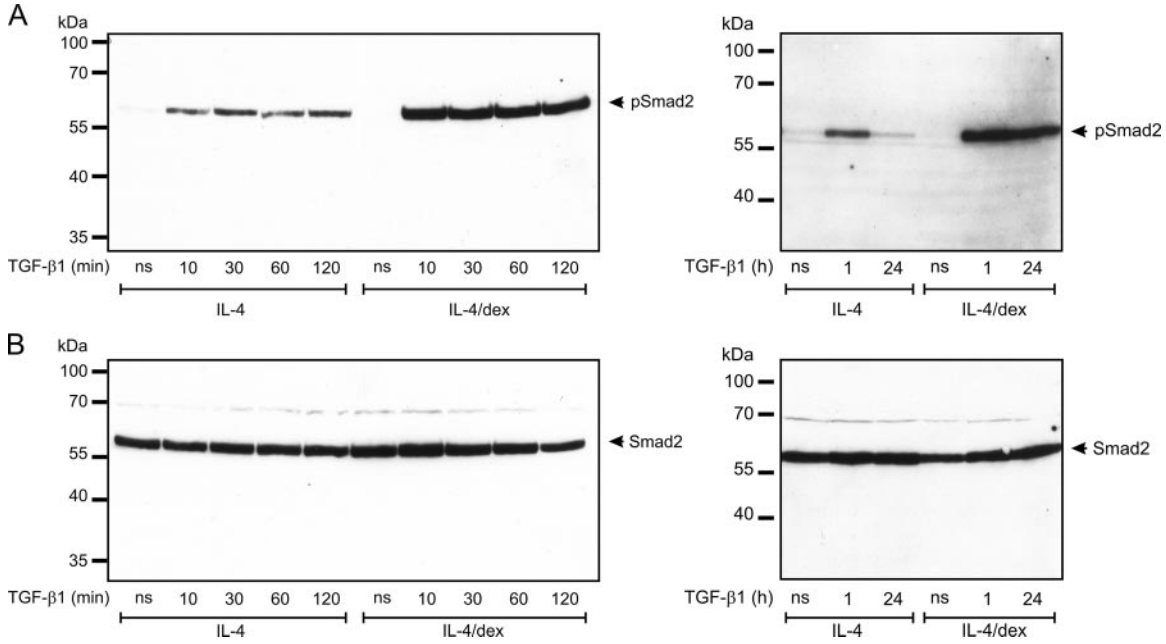

FIGURE 6. Analysis of Smad2 phosphorylation kinetics. Mature $M2_{IL\text{-}4/dex}$ were stimulated by TGF-β1 as indicated. The amounts of phosphorylated Smad2 (pSmad2) (*A*) and total Smad2 (*B*) were analyzed using Western blotting.

RT-PCR analysis revealed that the expression of IL17RB and Smad7 mRNA were not activated by TGF-β1 in control macrophages and in macrophages stimulated by each cytokine alone. In contrast, the expression of ID3 and OLR1 mRNA was up-regulated in response to TGF-β1 in control and macrophages and macrophages stimulated by M-CSF (Fig. 4*B*). This finding fits well with the residual surface expression of TGF-βRII in control and M-CSF-treated macrophages (Figs. 3*C* and 4*A*).

Stimulation of macrophages by dexamethasone alone or by dexamethasone in combination with either IL-13, M-CSF, or GM-CSF induced efficient up-regulation of mRNA expression of OLR1, ID3, and Smad7 in response to TGF-β1. In the case of IL17RB, significant up-regulation of expression was observed only in the case of stimulation by both IL-13 and dexamethasone, because IL-4 or IL-13 is required for induction of IL17RB expression (14).

Dexamethasone regulates the surface expression of TGF-βRII in a time- and dose-dependent manner

We next tested whether dexamethasone regulated the surface expression of TGF-βRII in a dose-dependent manner. Monocyte-derived macrophages were differentiated for 5 days in the presence of IL-4 with addition of gradually increasing concentrations of dexamethasone covering a range from 1×10^{-7} to 1×10^{-9} M. FACS analysis showed that the level of surface expression of TGF-βRII clearly depends on the concentration of dexamethasone in the medium (Fig. 5*A*). The surface expression of TGF-βRII was already significantly increased at a concentration of 1×10^{-8} M dexamethasone; this concentration is within the range of dexamethasone levels (4×10^{-9} to 2×10^{-8} M) that correspond to physiological cortisol levels in the serum of an adult individual (Fig. 5*A*). It should also be noted that the surface expression of TGF-βRII at a concentration of 1×10^{-8} M dexamethasone is only three times lower than the level of surface expression of TGF-βRII at the starting point of pharmacologically effective concentrations of dexamethasone (1×10^{-7} M) (Fig. 5*A*) observed, for example, during systemic GC therapy of rheumatoid arthritis patients (33).

We next investigated whether surface expression of TGF-βRII is an immediate effect of dexamethasone or whether it is a consequence of monocyte-to-macrophage differentiation in the presence of dexamethasone. Primary monocytes were cultivated for 1, 2, 3, 4, and 5 days in medium supplemented with IL-4 and 1×10^{-7} M dexamethasone or IL-4 alone. Surface expression of TGF-βRII was analyzed using flow cytometry. We observed that in $M2_{IL\text{-}4}$, the expression of TGF-βRII was reduced to background values within the first 3 days of culture (Fig. 5*B*). These data are in a good agreement with the previously described down-regulation of TGF-βRII expression during macrophage differentiation (22). In $M2_{IL\text{-}4/dex}$, however, the surface expression of TGF-βRII was slightly reduced after the first day of culture but increased continuously afterward, reaching a plateau on the 4th day of culture (Fig. 5*B*). The difference in surface expression of TGF-βRII between $M2_{IL\text{-}4}$ and $M2_{IL\text{-}4/dex}$ was already statistically significant on the first day of culture (Fig. 5*B*). These data indicate that the presence of GCs is required during the differentiation of monocytes to macrophages to maintain surface expression of TGF-βRII. Taken together, our results suggest that physiological concentrations of cortisol are sufficient for a TGF-β response in M2 macrophages. Therapeutic use of GCs may lead to enhanced surface expression of TGF-βRII and to an amplified response to TGF-β in M2 macrophages.

TGF-βRII induced by dexamethasone is functionally active

We next tested whether the increase in surface expression of TGF-βRII induced by dexamethasone leads to an increase in functional activity of the TGF-β receptor complex. As phosphorylation of Smad2 is an indication of TGF-β receptor activation, we analyzed Smad2 phosphorylation after stimulation of $M2_{IL\text{-}4}$ and $M2_{IL\text{-}4/dex}$ with TGF-β1. Western blot analysis of Smad2 phosphorylation was conducted using lysates of $M2_{IL\text{-}4}$ and $M2_{IL\text{-}4/dex}$, as well as of $M2_{IL\text{-}4}$ and $M2_{IL\text{-}4/dex}$ stimulated by TGF-β1 for 10, 30, 60, and 120 min (Fig. 6*A*). We found that the amount of pSmad2 after TGF-β1 stimulation was at least 10-fold higher in $M2_{IL\text{-}4/dex}$ than in $M2_{IL\text{-}4}$; this difference was already clearly visible after 10 min of

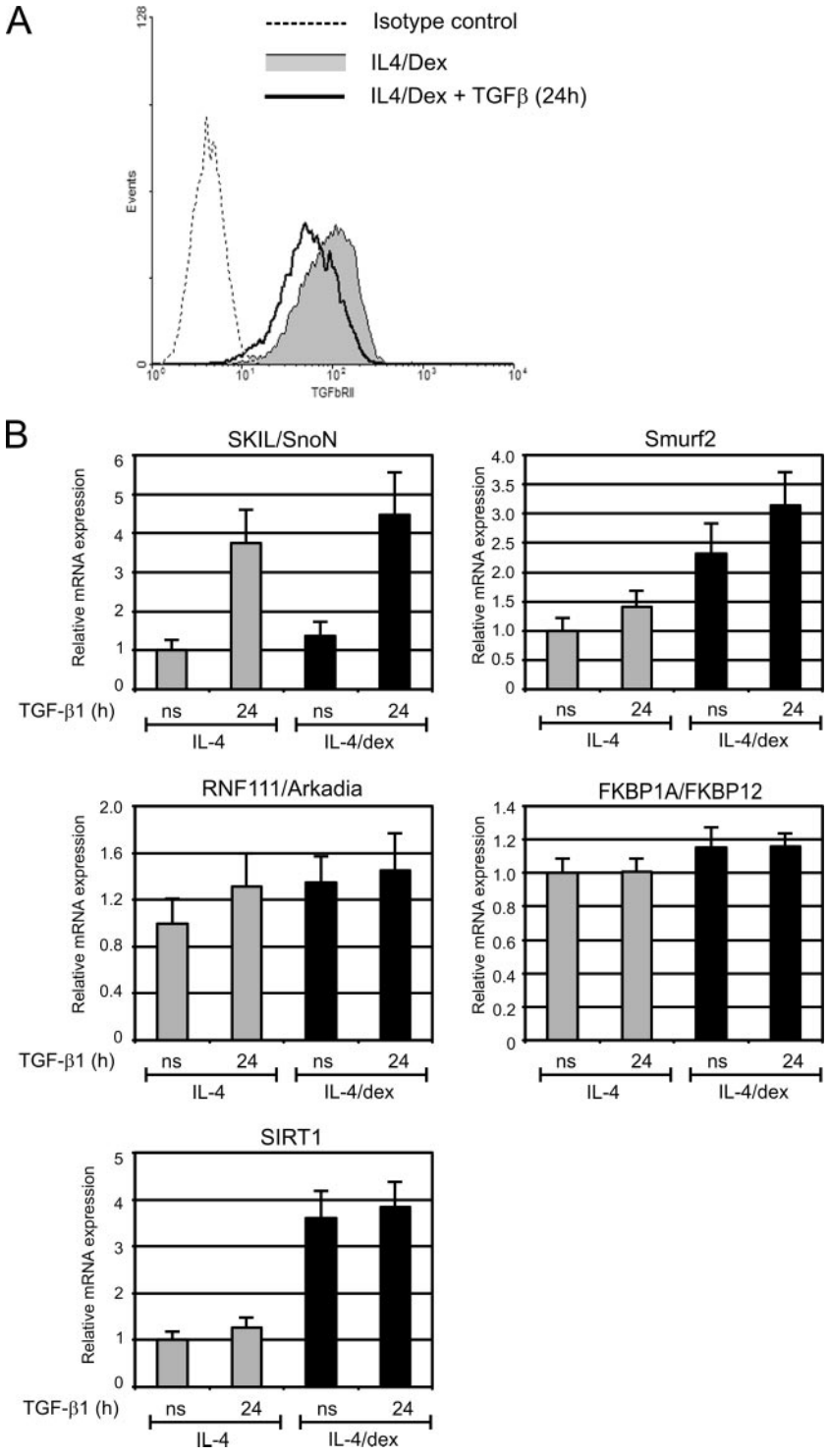

FIGURE 7. Analysis of feedback mechanism in M2 macrophages. *A*, Mature $M2_{IL\text{-}4/dex}$ were stimulated by TGF-β1 for 24 h. Surface expression of TGF-βRII in TGF-β1-stimulated and -nonstimulated macrophages was analyzed using flow cytometry. *B*, Macrophages were stimulated by IL-4 or IL-4/dexamethasone for 5 days and then by TGF-β1 as indicated. Analysis of Smurf2, Arkadia, SnoN, FKBP12, and SIRT1 mRNA expression was performed using real-time RT-PCR.

stimulation. The total amount of Smad2 remained at the same level in all macrophage populations and at all time points analyzed (Fig. 6*B*).

Although the level of Smad2 phosphorylation was significantly higher in $M2_{IL\text{-}4/dex}$, the kinetics of Smad2 activation was similar for both $M2_{IL\text{-}4}$ and $M2_{IL\text{-}4/dex}$ at the early time points of 10 min up to 2 h. As it is known that TGF-β1 induces Smad7 – a negative feed back regulator of TGF-β signaling (34) that was also up-regulated in our gene expression profiling experiments – we tested whether there were any differences between $M2_{IL\text{-}4}$ and $M2_{IL\text{-}4/dex}$ in TGF-β signaling at later time points. For this purpose, pSmad2 was analyzed in lysates of $M2_{IL\text{-}4}$ and $M2_{IL\text{-}4/dex}$ stimulated by TGF-β1 for 1 and 24 h (Fig. 6*A*). In the case of $M2_{IL\text{-}4}$, the amount of pSmad2 returned to almost baseline levels after 24 h despite continued TGF-β1 stimulation, indicating an efficient negative feedback mechanism. In $M2_{IL\text{-}4/dex}$, however, the amount of pSmad2 after 24 h of continued TGF-β1 stimulation was similar to the amount of pSmad2 observed at 1 h of stimulation (Fig. 6*A*).

To understand the mechanisms underlying prolonged TGF-β signaling in dexamethasone-stimulated macrophages, we investigated components of the feedback machinery in macrophages stimulated by IL-4 or IL-4/dexamethasone. As already described

earlier in this paper, Smad7 mRNA expression is strongly up-regulated in TGF-β1-activated $M2_{IL\text{-}4/dex}$. mRNA expression of other components of the feedback: Arkadia, SnoN, Smurf1, Smurf2, and FKBP12 were not significantly altered by dexamethasone according to our microarray data (data not shown). Real-time RT-PCR analysis showed no differences in expression of Arkadia, FKBP12, and SnoN mRNA between $M2_{IL\text{-}4}$ and $M2_{IL\text{-}4/dex}$ with or without TGF-β1 stimulation (Fig. 7*B*), whereas expression of Smurf2 mRNA was slightly increased under dexamethasone stimulation. We proposed that if negative feedback mechanism is functional in $M2_{IL\text{-}4/dex}$, the amount of TGF-βRII on the cell surface must be reduced upon TGF-β1 stimulation. Indeed, FACS analysis revealed that surface expression of TGF-βRII protein was reduced by 30% in $M2_{IL\text{-}4/dex}$ stimulated by TGF-β1 for 24 h (Fig. 7*A*), indicating that negative feedback mechanism is active, but not sufficient to completely remove TGF-βRII from the cell surface.

Next, we analyzed the expression of mRNA of SIRT1, a histone deacetylase that was shown to deacetylate Smad7 and facilitate its degradation (35), a mechanism that attenuates Smad7-mediated effects. Real-time RT-PCR analysis revealed that the expression of SIRT1 was increased 3-fold in $M2_{IL\text{-}4/dex}$ as compared with $M2_{IL\text{-}4}$ (Fig. 7*B*). This result suggests that GC-induced up-regulation of SIRT1 expression leads to insufficient activity of Smad7-based negative feedback.

These data indicate that TGF-βRII expressed on the surface of dexamethasone-treated macrophages is functionally active as shown by phosphorylation of Smad2 in response to TGF-β1 stimulation. Reduction of TGF-βRII surface expression in $M2_{IL\text{-}4/dex}$ upon TGF-β1 stimulation indicates that negative feedback mechanism is functional in these macrophages, but its activity is not sufficient to fully inactivate TGF-β signaling.

Discussion

In this study, we demonstrate that mature macrophages require the presence of GC to become permissive to the effects of TGF-β. In $M2_{IL\text{-}4/dex}$, TGF-β activates a multistep gene expression program featuring transcriptional regulators, immune modulators, and genes associated with lipid processing/atherosclerosis.

We searched for the molecular mechanisms underlying the GC-mediated differences in TGF-β responses of mature macrophages. We found that GC induced the expression of TGF-βRII on the cell surface of macrophages when used alone or in combination with IL-4, IL-13, M-CSF, or GM-CSF. It is well established that circulating monocytes express high numbers of TGF-β receptors on the cell surface (22, 36). However, the ability of differentiated macrophages to respond to TGF-β, is a matter of discussion (26). In earlier studies it was shown that stimulation of freshly isolated monocytes by LPS or IFN-γ for 18 h reduced binding of radiolabeled TGF-β (21), whereas IL-4, GM-CSF, M-CSF, TNF-α, and IL-1 did not cause this effect (21, 37). In a later paper, Ashcroft (22) claimed that the ability of macrophages to respond to TGF-β is lost upon differentiation of monocytes into macrophages due to the loss of TGF-β receptor surface expression. In our experimental system, we observed that monocytes differentiated into macrophages in the presence of IL-4 lose the surface expression of TGF-βRII within 72 h. We found that relatively low concentrations of dexamethasone (1×10^{-8} M) corresponding to physiological levels of cortisol in human plasma suffice to maintain detectable TGF-βRII on the surface of M2 macrophages, at a level similar to that observed on freshly isolated monocytes. Moreover, pharmacological concentrations of dexamethasone lead to enhanced surface expression of TGF-βRII that might render these M2 macrophages even more susceptible to the effects of TGF-β. It has been described previously that dexamethasone may exert significant effects on the subcellular localization of proteins, such as viral glycoproteins (38), β-catenin (39), and ZO-1 (40). The effect of dexamethasone on ZO-1 resembles its effect on TGF-βRII, as dexamethasone does not affect ZO-1 protein expression, but increases its surface expression (40). The mechanisms of these GC effects are still largely unknown. Regarding cell sorting of viral glycoproteins, de novo mRNA and protein expression are required to generate dexamethasone-mediated effects (38).

In addition to increased surface expression of TGF-βRII, $M2_{IL\text{-}4/dex}$ treated by TGF-β1 displayed prolonged Smad-mediated signaling. The main negative feedback mechanism of TGF-β signaling involves activation of I-Smads (Smad6 and Smad7), which block phosphorylation of R-Smads and drive TGF-βRII into degradation (41). We show here that Smad7 was indeed induced by TGF-β stimulation of $M2_{IL\text{-}4/dex}$ and that surface expression of the TGF-βRII in $M2_{IL\text{-}4/dex}$ was consequently reduced by 30% after 24 h of stimulation by TGF-β1, indicating the activity of negative feedback mechanism. In accordance with these findings, our microarray data and additional real-time RT-PCR analyses did not reveal significant differences in the expression of several components of the feedback machinery between $M2_{IL\text{-}4}$ and $M2_{IL\text{-}4/dex}$. The analysis of the expression of SIRT1, a newly recognized negative regulator of Smad7, showed that it is significantly up-regulated by dexamethasone. Therefore, we conclude that GC-mediated up-regulation of SIRT1 and active trafficking of the TGF-βR complex to the cell surface are responsible for prolonged Smad signaling in $M2_{IL\text{-}4/dex}$.

Activation of $M2_{IL\text{-}4/dex}$ by TGF-β leads to the development of a complex macrophage phenotype. During the early phase of TGF-β activation, *ID3* and *RGS1* were the most highly up-regulated genes in $M2_{IL\text{-}4/dex}$. ID3 is a known negative transcriptional regulator of the basic helix-loop-helix (bHLH) family that inhibits other bHLH transcription factors (42). ID3 has complex function in the immune and vascular systems including support of B cell immunity, dendritic cell differentiation, and angiogenesis (43, 44). RGS1 has been described as a negative regulator of G protein signaling in mononuclear cells (45). The functions of RGS1 in M2 macrophages are not known. It may, however, inhibit M2 macrophage migration in response to proinflammatory signals, a precondition for tissue remodeling, but also a clue to the development of fixed macrophages in various pathologies including the atherosclerotic plaque.

Besides *ID3*, early TGF-β response genes in M2 macrophages include well established targets of TGF-β, such as the AP-1 family member *JUNB* (27), the ETS-domain transcription factor *ELK3* involved in TGF-β-mediated suppression of LPS-induced NO synthase 2(NOS2) (46), and the runt-domain containing transcription factor *RUNX3*, necessary for TGF-β induced suppression of DC maturation (47). Induced expression of the vitamin D receptor provides an additional mechanism for the known synergistic antiproliferative effect of TGF-β and vitamin D as observed in myeloid leukemia cell lines (48). Transcription regulators *ELL2*, *TLE3*, and *BCoR* also up-regulated by TGF-β were implicated in the maintenance of undifferentiated cell states and in suppression of apoptosis (49–51). Transcription factors that belong to the group of late TGF-β response genes in M2 macrophages include another AP-1 family member, *FOS* (27), and *human myeloid nuclear differentiation Ag* implicated in inflammatory reactions and apoptosis and present in macrophages in atherosclerotic plaques (52, 53). We conclude that these transcription factors together with the R-Smads are responsible for the development of the macrophage phenotype observed after stimulation of $M2_{IL\text{-}4/GC}$ by TGF-β.

Two major groups of late TGF-β response genes were identified, i.e., genes involved in lipid metabolism and genes involved in immune modulation. The group of genes involved in lipid uptake, transport, and processing included *APOE* (17), *Lectin-like oxidized low density lipoprotein receptor-1* (*LOX* or *OLR1*) (29), *apolipoprotein C-II* (*APOC2*) (54), *sortilin-related receptor L* (*SORL1* or *LR11*), *LRPAP1*, and the ABC transporter *ABCG1*. It is well established that all these genes are involved in the regulation of cholesterol homeostasis and are implicated in the development of atherosclerosis (17, 55); in addition, macrophages within atherosclerotic plaques express APOE (56), OLR1 (57), and ABCG1 (58).

Besides *RGS1*, the genes *hepcidin*, *arachidonate-5-lipoxygenase-activating protein* (*ALOX5AP*), *TREM1*, and *IL17RB* are the most strongly up-regulated immune response-related genes found in TGF-β-activated $M2_{IL\text{-}4/dex}$. Hepcidin was originally described as an antimicrobial peptide, but was soon recognized to be important for retention of iron in hepatocytes and macrophages (59). The role of hepcidin in atherosclerotic diseases is still a matter of discussion. *ALOX5AP* and LTA_4 hydrolase, also induced in TGF-β-activated $M2_{IL\text{-}4/dex}$, are important in leukotriene synthesis, especially LTB_4 that is involved in formation of the atherosclerotic plaque and is produced mainly by plaque macrophages (60). Furthermore, ALOX5AP polymorphisms are associated with myocardial infarction and stroke (61). TREM1, an inflammation amplifier (62), and IL18R1, whose ligation leads to the activation of NFκB, as well as CXCR4, a CXC chemokine receptor responsible for CXCL12-mediated chemotaxis, support inflammation and atherosclerosis (63–65). Taking into consideration the genes of the lipid metabolism and immune modulation groups, it remains an open question whether the TGF-β-activated gene expression program in M2 macrophages would favor the development of atherosclerosis or contribute to the putative protective functions of TGF-β during atherosclerosis (66, 67).

In summary we established that mature macrophages retain the ability to respond to TGF-β if differentiated in the presence of GCs. In M2 macrophages, TGF-β1 induces the development of a phenotype featuring traits of macrophages found within an atherosclerotic lesion. Upon GC therapy, TGF-β effects on macrophages may be enhanced by further up-regulation of TGF-βRII.

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Disclosures

The authors have no financial conflict of interest.

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