

Transforming Growth Factor- β 1 Regulates the Expression of the High-Affinity Receptor for IgE on CD34⁺ Stem Cell-Derived CD1a⁺ Dendritic Cells *In Vitro*

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It has been reported that monocytes, Langerhans cells (LC) and other dendritic cells (DC) express the high-affinity receptor for IgE (Fc ϵ RI) in patients with atopic diseases. These cells may be instrumental in the control of the immune response and the allergic inflammation. In this context, transforming growth factor beta 1 (TGF- β 1) has been highlighted as a key cytokine involved in the mechanisms aimed to orchestrate tolerance and has been suggested as a candidate gene in atopic diseases. In this report, we investigate the putative role of TGF- β 1 in the regulation of Fc ϵ RI on cord blood CD34⁺ stem cell-derived CD1a⁺ DC (CD34-derived CD1a⁺ DC). Kinetic experiments show that Fc ϵ RI spontaneously appears on the surface of CD1a⁺ DC, but decreases when exogenous TGF- β 1 is added at high doses (10 ng per mL) or when endogenous TGF- β 1 is neutralized in the culture conditions. In contrast, low-dose TGF- β 1 (0.5 ng per mL) stabilizes surface Fc ϵ RI expression on DC. Increasing TGF- β 1 concentrations leads to the generation of LC-like DC showing an augmentation in stimulatory capacity towards allogeneic T cells. In view of these data, a picture emerges that Fc ϵ RI⁺ on DC is finely modified by the TGF- β 1 concentration in the microenvironment and could be of primary relevance in the context of atopic diseases.

Key words: atopy/CD34⁺/cord blood/dendritic cells/IgE receptors/stem cells/TGF- β 1
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The identification of the high-affinity receptor for IgE (Fc ϵ RI) on the surface of professional antigen-presenting cells (APC) from the myeloid lineage such as monocytes (Mo) and dendritic cells (DC) in the blood or tissue of atopic individuals lead to a new view of cellular distribution and functionality of this structure (Bieber, 1997). Interestingly, in contrast to basophils and mast cells, Fc ϵ RI surface expression on Mo and DC is highly variable. Therefore, factors regulating the Fc ϵ RI surface expression are of primary interest since they are crucial for our understanding of the physiological and pathophysiological role of this structure.

In the last decade, much progress has been made in the development of *in vitro* models for the generation of large numbers of DC. The first strategy is based on the generation of so-called Mo-derived DC. Alternatively, DC can be derived from CD34⁺ stem cells obtained from human adult or cord blood. Detailed analysis revealed that under appropriate conditions, CD34⁺-derived cells have the capacity to acquire distinct properties of DC subtypes (Caux *et al*, 1992;

Banchereau and Steinman, 1998). In previous studies, a varying spontaneous surface expression of Fc ϵ RI on *in vitro* generated CD1a⁺ DC has been reported (Geiger *et al*, 2000). For Mo-derived CD1a⁺ DC it has been shown recently that intracellular α -chain (Fc ϵ RI α) accumulates during DC differentiation, whereas the expression of γ -chains (Fc ϵ RI γ) mandatory for surface expression, is downregulated. It is low or negative in DC from normal donors lacking surface Fc ϵ RI (Fc ϵ RI⁻ DC). In contrast, CD1a⁺ DC from atopics show significant Fc ϵ RI γ expression and display surface Fc ϵ RI (Fc ϵ RI⁺ DC) (Novak *et al*, 2003).

Recently, it has been demonstrated that transforming growth factor- β 1 (TGF- β 1) contributes to the generation of Fc ϵ RI bearing DC from circulating monocytes (Reich *et al*, 2001). TGF- β 1 is a multifunctional growth factor belonging to a superfamily of regulatory proteins and it has been suggested that variations of TGF- β in the peripheral blood and affected tissues influence the development and course of atopic diseases (Lee *et al*, 2000; Arkwright *et al*, 2001). Furthermore, genetic studies aiming to detect atopy-related single nucleotide polymorphisms of the TGF- β 1 gene have suggested this cytokine as a putative candidate gene for atopic conditions since they could encode for low-producer individuals (Arkwright *et al*, 2001; Pulleyn *et al*, 2001). These observations prompted us to investigate the putative role of TGF- β 1 in the regulation of Fc ϵ RI on CD34-derived CD1a⁺ DC generated *in vitro* from CD34⁺ stem cells and we evaluated their functional characteristics.

Abbreviations: 7AAD, 7-aminoactinomycin-D; APC, antigen-presenting cells; DC, dendritic cells; FCS, fetal calf serum; Fc ϵ RI, high-affinity IgE receptor; Fc ϵ RI α , alpha-chain of the high-affinity IgE receptor; Fc ϵ RI γ , gamma-chain of the high-affinity IgE receptor; LC, Langerhans cells; MHC I, major histocompatibility complex I; MHC II, major histocompatibility complex II; PE, phycoerythrin; TGF- β 1, transforming growth factor beta 1

Results

TGF- β regulates the generation of CD34-derived CD1a⁺ DC during *in vitro* In a first attempt, we evaluated the effect of different concentrations of TGF- β 1 on CD34⁺ stem cell cultures. By this, we could show that addition of TGF- β 1 both at low (TGF- β 1^{low}) and high (TGF- β 1^{high}) concentrations significantly increased the generation of CD34-derived CD1a⁺ DC compared to control cultures (CON) without any TGF- β 1 supplementation (CON: 23.90% \pm SEM 2.02, TGF- β 1^{low}: 56.24% \pm SEM (2.81 and TGF- β 1^{high}: 63.4% \pm SEM 3.85). It has been shown previously that a high TGF- β 1 level sustains hematopoietic immaturity, whereas neutralization of endogenous and autocrine TGF- β 1 sustains undifferentiated cells (Ottmann and Pelus, 1988; Fortunel *et al*, 2000). Moreover, culture condition under TGF- β 1^{high} did not only decelerate cell proliferation as indicated by lower total cell number in our system (Fig 1A) but also decelerated the differentiation of CD34⁺ stem cells. In contrast to CD34-derived CD1a⁺ DC generated under control conditions, CD34⁺ stem cells were still detectable in cultures with TGF- β 1^{low} and TGF- β 1^{high} after 9 d (Fig 1B). From these first series of experiments, we conclude that increasing amounts of TGF- β 1 enhance the generation of CD34-derived CD1a⁺ DC but decelerate cell proliferation and sustain cell immaturity.

IgE and low concentrations of TGF- β 1 stabilize FcεRI on *in vitro* generated CD1a⁺ DC To investigate the regulation of the FcεRI expression of CD34-derived CD1a⁺ DC during their *in vitro* differentiation, cells were subjected to detailed flow cytometric analyses. FcεRI appeared on the cell surface of CD34-derived CD1a⁺ DC under all three investigated conditions and peaked around day 5. The receptor expression stabilized at high level under control condition and TGF- β 1^{low}. In contrast, FcεRI was rapidly downregulated on CD34-derived CD1a⁺ DC generated with TGF- β 1^{high} and on day 9 only few cells bearing FcεRI could be detected (Fig 2A). In order to create an atopy-like microenvironment, CD34-derived CD1a⁺ DC were generated in the presence of IgE since it has been shown that its binding to FcεRI α may contribute to the upregulation of the receptor on APC (Reischl *et al*, 1996; Borkowski *et al*, 2001). These culture conditions lead to a further significant increase of

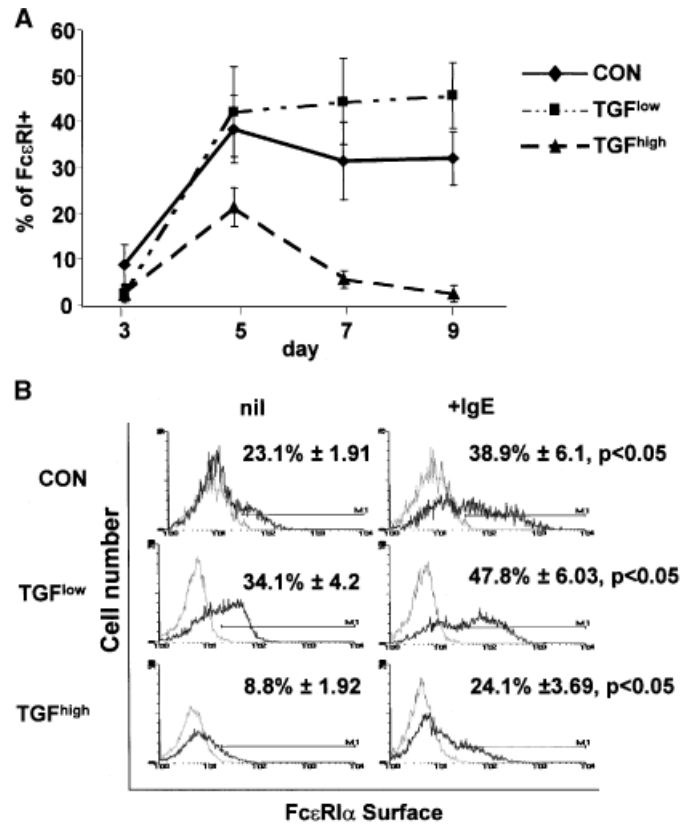


Figure 2
FcεRI expression of CD34-derived CD1a⁺ DC during differentiation and on day 9. (A) FcεRI regularly appears on differentiating CD34-derived CD1a⁺ DC under all conditions peaking around day 5 and stabilizing under control condition (CON) and TGF- β 1^{low} (n = 7). (B) Addition of IgE resulted in an increased FcεRI expression of CD34-derived CD1a⁺ DC under all culture conditions. On day 9, FcεRI could even be demonstrated on CD34-derived CD1a⁺ DC generated with TGF- β 1^{high} (n = 5). Mean values \pm SEM of five experiments are shown in the upper right corner of the histogram including p values.

the FcεRI expression of CD34-derived CD1a⁺ DC (Fig 2B). Thus, FcεRI is constitutively expressed on the surface of CD34-derived CD1a⁺ DC during their cell differentiation from CD34⁺ stem cells, but this expression is subjected to further regulatory effects depending on the presence of TGF- β 1 and IgE in the microenvironment.

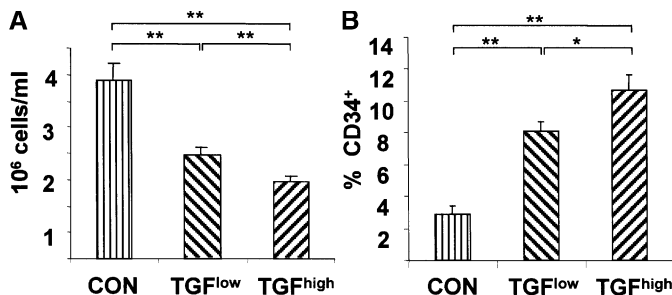


Figure 1
TGF- β 1 decelerates cell proliferation and sustains cell immaturity. (A) The highest cell yield was detected in control culture (CON). TGF- β 1^{low} and TGF- β 1^{high} decreased total cell proliferation with the lowest cell yield under high TGF- β 1^{high} (n = 26). (B) In contrast to control culture (CON), CD34^{pos} stem cells were still detectable on day 9 in cultures with TGF- β 1^{low} and TGF- β 1^{high} (n = 11).

TGF- β 1 regulates the expression of FcεRI subunits FcεRI on APC differs from the tetrameric FcεRI found on the effector cells of anaphylactic reactions by lacking the β -chain and consisting only of an IgE-binding α -chain and two disulfide linked γ -chain (Turner and Kinet, 1999). In addition, a high amount of FcεRI γ , preferably found on APC of atopic individuals, is mandatory for the FcεRI surface expression (Maurer *et al*, 1994; Maurer *et al*, 1996). Thus, to arrive at the exact structure of FcεRI on CD34-derived CD1a⁺ DC and the protein level of the respective FcεRI α and FcεRI γ chains on CD34-derived CD1a⁺ DC, we performed extracellular and intracellular flow cytometric analyses in combination to semiquantitative PCR experiments. The latter showed comparable amounts of FcεRI α and FcεRI γ transcripts under all three cell culture conditions used (Fig 3A), whereas FcεRI β -chain remained undetectable (data not shown). Importantly,

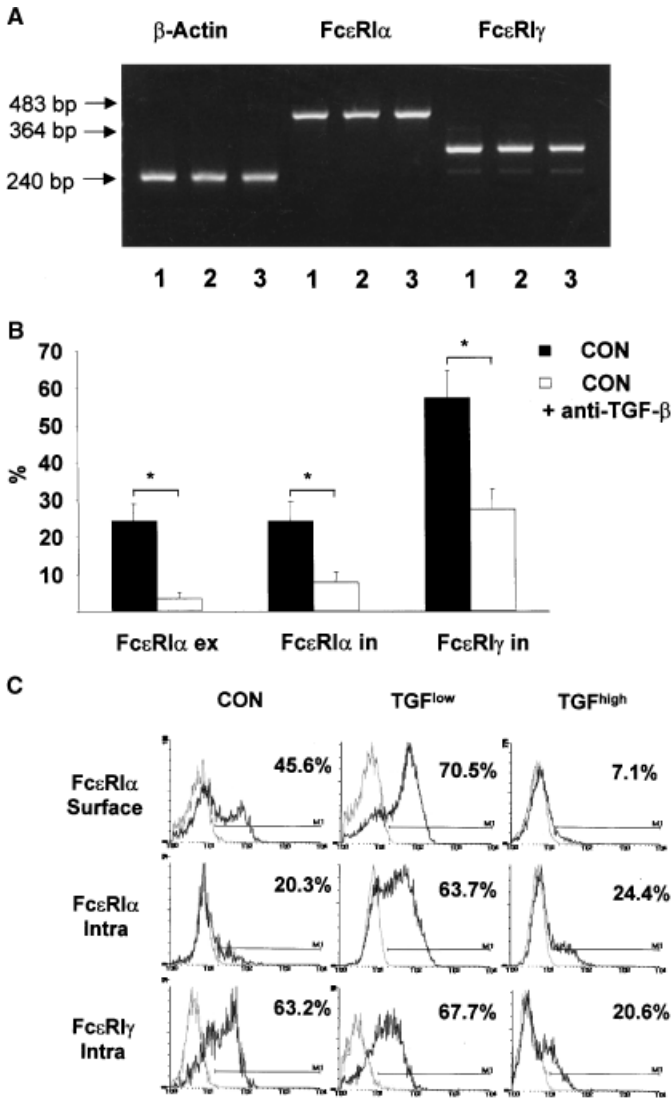


Figure 3
Alteration of FcεRIα and FcεRIγ in response to different concentrations of TGF-β1. (A) Representative results of RT-PCR of highly enriched CD1a⁺ cells confirming the presence of FcεRIα and FcεRIγ (1 = control condition, 2 = TGF-β1^{low}, 3 = TGF-β1^{high}, n = 3) (B) Blocking endogenous TGF-β with a monoclonal anti-TGF-β antibody in control condition culture leads to a decreased surface FcεRI expression on CD34-derived CD1a⁺ DC on day 9 accompanied by a strong decrease of intracellular FcεRIα and FcεRIγ (n = 7) Furthermore, control experiments revealed that supplementation of irrelevant IgG1 antibody did not alter surface expression of FcεRI (data not shown). (C) In contrast to CD34-derived CD1a⁺ DC generated under control culture (CON) and TGF-β1^{low}, TGF-β1^{high} downregulated FcεRI surface expression on CD34-derived CD1a⁺ DC by decreasing the intracellular pool of FcεRIγ. Intracellular FcεRIα was present under all conditions with the strongest detection in CD34-derived CD1a⁺ DC generated with TGF-β1^{low}. Representative histograms of 12 experiments are shown.

transcripts of tryptase as a control for contaminating mast cells or basophils could not be detected (data not shown).

Since CD34-derived CD1a⁺ DC were generated in FCS, which served as a source of TGF-β1, we determined the level of endogenous TGF-β1 in the cell culture supernatant of control condition on day 9. As expected, an average of 0.03 ng per mL (SEM ± 0.01, n = 6) TGF-β1 could be detected. This corresponds to the amount of TGF-β1 from FCS supplementation of culture medium and excludes and

TGF-β1 producing source such as platelets in our cell culture system. Complete neutralization of endogenous TGF-β1 leads to rapid downregulation of the FcεRI surface expression on day 9. Interestingly, both receptor subunits, intracellular FcεRIα and FcεRIγ were hardly detectable and only trackable in decreased amounts after TGF-β neutralization (Fig 3B). Furthermore, control experiments revealed that supplementation of irrelevant IgG1 antibody did not alter surface expression of FcεRI (data not shown).

CD34-derived CD1a⁺ DC generated under control conditions displayed a moderate FcεRI surface expression, whereas the highest FcεRI surface expression and intracellular FcεRI pool of the α-chain was detectable on CD34-derived CD1a⁺ DC generated with TGF-β1^{low} (Fig 3C). Although the intracellular pool of FcεRIγ was elevated in CD34-derived CD1a⁺ DC cultured under control conditions and TGF-β1^{low}, only low levels of FcεRIγ were detectable in CD34-derived CD1a⁺ DC generated in TGF-β1^{high} (Fig 3C).

Based on these cumulative data, we can conclude that the presence of TGF-β1 in the microenvironment surrounding CD34-derived CD1a⁺ DC is a crucial factor in the control of surface expression of FcεRI on CD34-derived CD1a⁺ DC.

TGF-β1 concentration as the limiting factor for induction of FcεRI bearing Langerhans cells (LC)

Several types of CD34-derived CD1a⁺ DC have been identified in the human immune system whereas TGF-β1 is critical for the generation of LC *in vitro* (Strobl *et al*, 1996). Thus we investigated the amount of the CD1a⁺ LC subset under different culture conditions detected by the expression of Langerin, a lectin receptor specific for LC (Valladeau *et al*, 2000). Whereas under control conditions, we were not able to detect LC, these cells could be generated in TGF-β1^{low}. The highest number of LC among CD34-derived CD1a⁺ DC could be detected under TGF-β1^{high}. Interestingly, under TGF-β1^{low} a remarkable amount of LC were FcεRI⁺ (Fig 4).

Thus, we can conclude that TGF-β1 at defined concentrations (TGF-β1^{low}) leads to the generation of FcεRI⁺ LC.

TGF-β derived CD34-derived CD1a⁺ DC display distinct phenotypical and functional characteristics

To investigate the nature of DC generated under control condition, we performed morphological and detailed phenotypical analyses in comparison to CD34-derived CD1a⁺ DC generated under TGF-β1 supplementation. As reported earlier, addition of TGF-β1 leads to the formation of clusters (Riedl *et al*, 2000b) whereas a concomitant increase of E-cadherin—a homophilic adhesion molecule involved in the cluster formation of LC (Riedl *et al*, 2000a)—could be observed (Fig 5A). Although we could not find a significant difference in expression of DC-SIGN/CD209, we could detect on CD34-derived CD1a⁺ DC generated under control condition an increased expression of CD11b and mannose receptor (CD206) that are not only expressed by macrophages but also related to DC of non-LC type and are known to be expressed on so-called inflammatory dendritic epidermal cells (IDEC) that are usually found in lesional sites of the skin of atopic eczema patients (Sallusto *et al*, 1995; Jaksits *et al*, 1999; Wollenberg *et al*, 2002) (Fig 5). In addition CD14 could be detected in approximately equal amounts on CD1a positive cells under all conditions (Fig 5A). Furthermore the

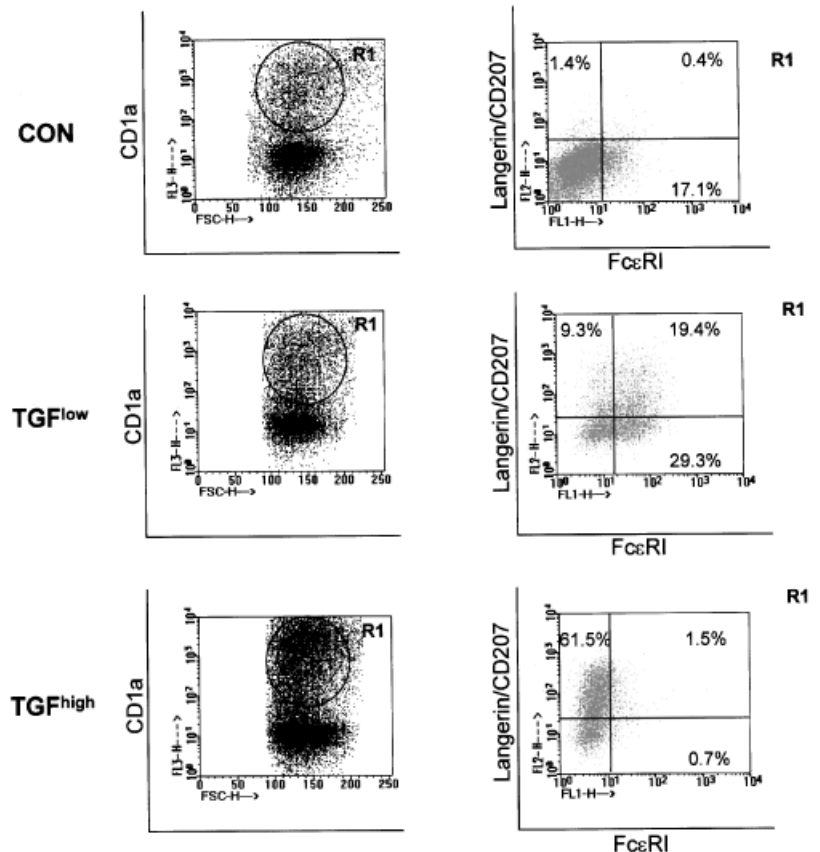


Figure 4
High amounts of FcεRI⁺ LC were generated with TGF-β1^{low}. The generation of CD34-derived CD1a⁺ DC and LC depended on exogenous TGF-β1 supplementation. The highest amounts of Langerin⁺ LC were generated under TGF-β1^{high} followed by LC generated with TGF-β1^{low}. Under control culture (CON) virtually no Langerin⁺ LC could be detected. A high percentage among the LC population expressed FcεRI especially in cultures with TGF-β1^{low} (n = 5).

addition of exogenous TGF-β1 augmented the expression of major histocompatibility complex (MHC) class I and II complexes and costimulatory molecules, but did not result in the final maturation of CD34-derived CD1a⁺ DC indicated by lack of CD83, a marker for mature DC (Fig 5B) (Zhou and Tedder, 1996).

To investigate the influence of TGF-β1 on the functional properties of FcεRI⁺ CD34-derived CD1a⁺ DC, we tested their stimulatory activity in an allogeneic system. As shown in Fig 6, the addition of TGF-β1 leads to generation of CD34-derived CD1a⁺ DC, which display a significant increased allogeneic stimulatory activity.

Discussion

TGF-β1 is a pleiotropic regulator of the hematopoiesis, which displays a growth inhibitory effect on early progenitor cells and influences distinctly the differentiation of CD34-derived CD1a⁺ DC (Fortunel *et al*, 2000).

In this study, we investigated the involvement of TGF-β1 in the regulation of the FcεRI α- and γ-chains and the differentiation of FcεRI bearing subsets of CD34-derived CD1a⁺ DC. We uncovered that especially the intracellular FcεRIγ pool, which is mandatory for sufficient FcεRI surface expression, is distinctly adjusted by the amount of TGF-β1, whereas the regulation of FcεRIγ subunits occur at the protein but not at the mRNA level suggesting that TGF-β is involved in the regulation of the γ-chain at a post-transcriptional point. Another group reported similar findings of γ-chain regulation in Mo-derived macrophage where infection

of the cells with HIV lead to downregulation of γ-chain on protein level but did not alter the mRNA expression (Kedzierska *et al*, 2002). Just recently, another group reported that TGF-β1 suppressed expression of Fcγ-receptors on human myeloid cells by downregulating the γ-chain, which is identical to FcεRIγ (Ra *et al*, 1989; Tridandapani *et al*, 2003).

At early differentiation stages, FcεRI is constitutively expressed on the cell surface of the differentiating CD34-derived CD1a⁺ DC. Later on, low or moderate TGF-β1 levels facilitate the differentiation of FcεRI bearing CD34-derived CD1a⁺ DC subtypes such as Langerin⁺ LC and Langerin⁻ CD34-derived CD1a⁺ DC. In contrast, the addition of TGF-β1 at high doses and the total elimination of TGF-β from the surrounding micromilieu prevent the generation of FcεRI⁺ CD34-derived CD1a⁺ DC subtypes during further differentiation and accelerate the differentiation of FcεRI⁻ CD34-derived CD1a⁺ DC such as LC and Langerin⁻ CD34-derived CD1a⁺ DC. TGF-β1 seems to play a major role in the regulation of FcεRI since the generation of the different FcεRI⁺ DC subtypes in combination with a distinct intracellular FcεRIα and FcεRIγ pool were influenced by addition of exogenous TGF-β1. As we used a TGF-β blocking antibody which was directed against TGF-β1, β2, and β3, we cannot exclude that downregulation of FcεRI results from an cooperative effect of all TGF-β isoforms.

Even though increasing evidence arises that TGF-β1 plays a pivotal role in atopic diseases, its influence on the expression of the high-affinity receptor for IgE on human APC has not been investigated in detail before. In the past, studies have shown that atopic diseases such as atopic

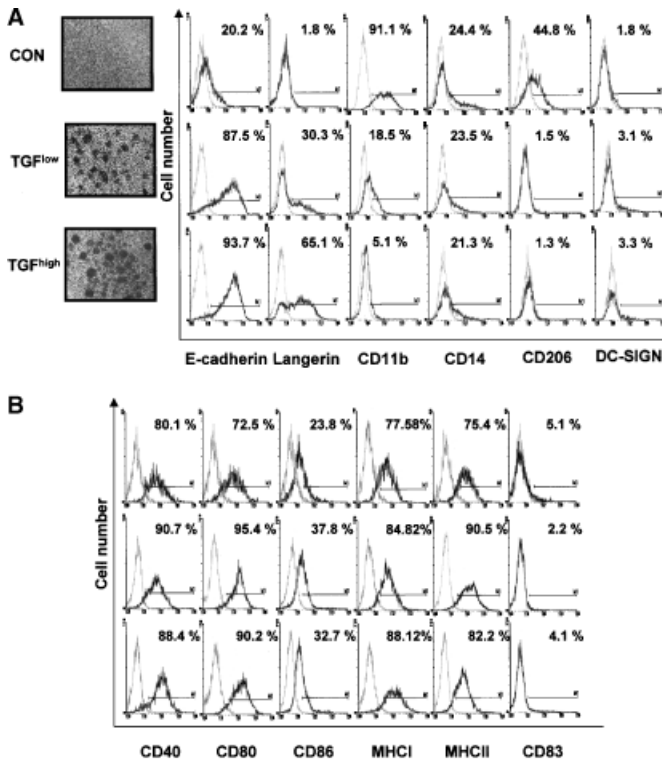


Figure 5
Phenotypical and morphological changes induced by TGF- β 1. (A) Representative histograms of E-cadherin, Langerin, CD11b, CD14, CD206 and DC-SIGN on CD34-derived CD1a⁺ DC (n = 5). (B) Representative phase contrast microscopic images of cells cultured with control condition, TGF- β 1^{low} and TGF- β 1^{high} and representative histograms co-stimulatory molecules and MHC class I and II complex, which were up-regulated through addition of exogenous TGF- β 1 (TGF- β 1^{low}, TGF- β 1^{high}). Note the enhanced cluster formation in cultures supplemented with exogenous TGF- β .

dermatitis go along with a low TGF- β 1 producing phenotype (Lee *et al*, 2000; Arkwright *et al*, 2001). These findings are largely compatible with recent data, which predict that in some instances defects in the TGF- β 1 production contribute to human diseases such as allergic hypersensitivity disorders (Sugai *et al*, 2003). These are characterized by an excessive IgE serum level in combination with an enhanced Fc ϵ RI surface expression on DC in the peripheral blood and tissues. Further on, it has been shown that TGF- β is largely involved in the regulation of the IgE synthesis itself and displays a downregulatory function on IgE serum levels (Wu *et al*, 1992; Sugai *et al*, 2003). In healthy, non-atopic individuals, high TGF- β serum levels prevail. The epidermal skin lesions of atopic dermatitis are characterized by high numbers of Fc ϵ RI-bearing LC and another Fc ϵ RI bearing DC subtype, the so-called IDEC. In contrast in the skin of healthy, non-atopic individuals IDEC are completely absent and Fc ϵ RI⁻ LC attend in the epidermis (Wollenberg *et al*, 1995). In view of the data presented here, low or moderate TGF- β 1 producing phenotype which are a pathognomonic feature of atopic individuals could promote the development of Fc ϵ RI⁺ DC subtypes *in vivo*. Since Fc ϵ RI appears on the cell surface of CD34-derived CD1a⁺ DC during differentiation, IgE as a receptor stabilizing mechanism via Fc ϵ RI α binding (Borkowski *et al*, 2001) might further contribute to the high Fc ϵ RI expression on DC in atopic indi-

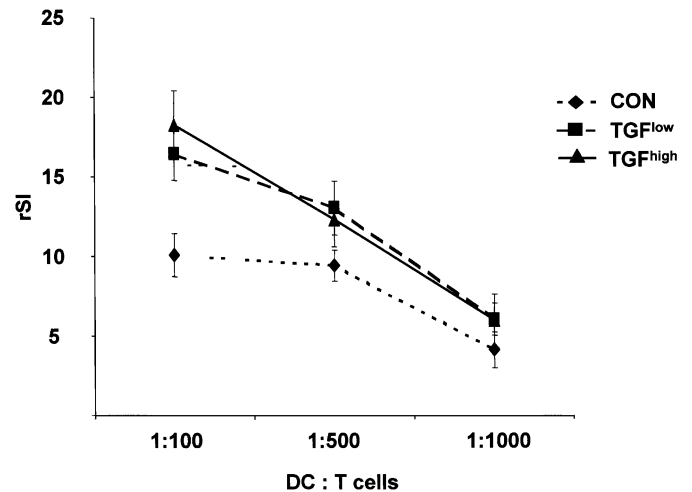


Figure 6
TGF- β leads to enhanced allogeneic stimulatory capacity of CD34-derived CD1a⁺ DC. DC were co-cultured with autologous or allogeneic T cells for 5 d as described in Material and Methods. CD34-derived CD1a⁺ DC generated in the presence of exogenous TGF- β 1 (TGF- β 1^{low}, TGF- β 1^{high}) lead to an increased stimulatory capacity towards allogeneic T cells (n = 5).

viduals, whereas in a non-atopic environment regular TGF- β levels could inhibit Fc ϵ RI surface expression on DC. Together, these components might trigger the generation of the pathophysiologically relevant Fc ϵ RI⁺ DC subtypes, which might perpetuate the course of pro-inflammatory and allergic processes of atopic diseases by their high antigen-presenting and stimulatory capacities *in vivo*.

Whether the wide margin of fluctuation of active TGF- β 1 levels in the human cord blood might bias or prevent selectively the development of these potent players in the atopic game *in vivo* and, therefore, serve as a predictive factor for the generation of atopic diseases later in life, remains to be elucidated.

Material and Methods

Reagents Phycoerythrin (PE)-labeled T6RD1 (IgG1, Beckman Coulter, Krefeld, Germany), unlabeled IOT6a (IgG1, Beckman Coulter) and Cy-Chrome-labeled HI149 (IgG1, Beckton Dickinson (BD), Heidelberg, Germany) recognize CD1a. Fc ϵ RI was detected by mAb 22E7 (IgG1) directed against the α -chain not interfering with the IgE binding site; mAb 4D8 (IgG2b) is directed against the γ -chain. These antibodies were prepared and characterized as described elsewhere (Riske *et al*, 1991; Schoneich *et al*, 1992); mAb IOT2b (IgG1) directed against HLA-DR, mAb M5E2 (IgG2a) directed against CD14, mAb BEAR-1 (IgG1) detecting CD11b and mAb HB15a detecting CD83 (IgG2b) were purchased from Immunotech, Marseille, France. MAb DCN46 (IgG2b) against DC-SIGN/CD209, mAb 19 (IgG1) detecting CD206 and mAb B70/B7-2 (IgG1) directed against CD86 and mAb 5C3 (IgG1) directed against CD40 (IgG1) were from BD. Mab. BB-1/B7 (IgG1) reacting with CD80 and PE-labeled 8G12 (IgG1) directed against CD34 were from BD, purified and PE-labeled mAb DCGM4 (IgG1) recognizes LC-specific Birbeck Granules (BG)-associated lectin Langerin/CD207 (Immunotech). MAb 67A4 (IgG1) detecting E-cadherin was purchased from Beckman-Coulter. MOPC-21 (IgG1, Sigma, Deisenhofen, Germany), mAb 1D11 is directed against TGF- β 1, - β 2, - β 3 (IgG1, R&D Systems, Minneapolis, Minnesota). MOPC-21 (IgG1), UPC10 (IgG2a), MOPC-141 (IgG2b) all from Sigma and IgG1RD1 (Beck-

man-Coulter) were used as appropriate isotype controls. Fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse (GaM/FITC) antibody was from Jackson Laboratories (West Grove, Pennsylvania). Sheep anti-mouse coated magnetic beads (M-280) were obtained from Dynal (Oslo, Norway). Normal mouse serum for blocking purposes, 7-aminoactinomycin-D (7AAD) and 2-mercaptoethanol were from Sigma. Human myeloma IgE (PS) was obtained from Calbiochem (Bad Soden, Germany). Heat-inactivated fetal calf serum (FCS), antibiotics/antimycotics and RPMI-1640 with Glutamax were all from Invitrogen (Carlsbad, California). Granulocyte macrophage colony stimulating factor (GM-CSF) was purchased from Novartis Pharma (Nürnberg, Germany), tumor necrosis factor (TNF- β), stem cell factor (SCF), Flt-3-ligand (FL3) and TGF- β 1 were purchased from R&D Systems.

Purification of CD34⁺ stem cells Cord blood was obtained from vaginal or cesarean deliveries after informed written consent from patients according to local Medical Ethics Committee of the University of Bonn, Germany and the study was performed according to Declaration of Helsinki Guidelines. After the blood was diluted three times with RPMI-1640 (Invitrogen), 25 mL of suspended cells was overlaid on 15 mL of Lymphoprep (Nycomed, Oslo, Norway). peripheral blood mononuclear cells (PBMC) were isolated as interface cells after density gradient centrifugation (20 min at 900 \times g at room temperature). To reduce residual platelets, the recovered cells were washed twice in PBS and recovered in PBS supplemented with 1% BSA and 5 mmol EDTA. Isolation of CD34⁺ cells was performed using magnetic microbeads coupled to anti-CD34 antibody and the AUTOMACS system (Milteny Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The enrichment of CD34⁺ cells was determined with an PE-conjugated anti-CD34 mAb and was more than 90% CD34⁺ cells.

Culture of CD34⁺ stem cells 4 \times 10⁴ purified CD34⁺ cells were cultured in 24-well plates (Corning, Acton, Massachusetts) with 0.5 mL RPMI-1640 with Glutamax (Invitrogen) supplemented with 10% heat inactivated FCS (Invitrogen), 1% antibiotics/antimycotics (Invitrogen) and 5 μ M ME (Sigma). Since it has been shown that combination of GM-CSF, TNF- α , SCF, FL3, and TGF- β 1 give rise to a great number of CD34-derived CD1a⁺ DC (Strobl *et al*, 1997), we used GM-CSF (300 IU per mL), TNF- β (50 IU per mL), SCF (10 ng per mL), and FL3 (10 ng per mL) in FCS supplemented medium as control culture conditions. We performed ELISA to analyze the amount of TGF- β 1 in the FCS charges used for medium supplementation. We detected an average TGF- β 1 concentration of 0.39 ng per mL (SEM \pm 0.09; n=3). Since we used 10% FCS supplemented medium we expected an endogenous TGF- β 1 concentration of 0.04 ng per mL. Hereafter, the baseline condition will be referred to as control. According to TGF- β 1 levels found in cord blood (0.89 ng per mL, SEM \pm 0.08, n=16) and to a previous study (Strobl *et al*, 1996), we defined low and high exogenous TGF- β 1 culture conditions as follows: low TGF- β 1 (TGF- β 1^{low}) with 0.5 ng per mL and high TGF- β 1 (TGF- β 1^{high}) with 10 ng per mL which were added to control conditions. The cells obtained will be referred to as CD34-derived CD1a⁺ DC.

Generation and culture of CD34-derived CD1a⁺ DC were maintained for 9 d for kinetic experiments. One-half of the medium was replaced with fresh cytokines after 4 and 8 d. To mimic an atopic environment CD34⁺ cells were cultured as described above in addition to 1 μ g per mL human myeloma IgE (every other days). Photographs were taken with a DMCRB microscope (Leica, Bensheim, Germany) using an HV-C20A digital camera (Hitachi, Dueseldorf, Germany) and Diskus software (Hilgers, Koenigswinter, Germany).

Enrichment of CD1a⁺ DC For RNA extraction, CD1a⁺ DC were enriched from the culture conditions using an anti-CD1a mAb bound to magnetic beads according to the manufacturer's protocol. Briefly, Dynabeads M-280 precoated with sheep anti-mouse mAb were incubated with anti-CD1a mAb IOT6. After washing procedures, DC cell suspensions were added to the bead sus-

pensions and incubated for 1 h at 4°C. CD1a⁺ cells bound to the beads were then purified by four rounds of washing procedures with the magnet, yielding purity greater than 98% as controlled by light microscopy.

Flow cytometric analysis and ELISA Double-staining experiments with saponin or digitonin for the detection of surface or intracellular distribution of FcεRI subunits were performed as described in detail (Kraft *et al*, 1998). For triple-staining experiments cells were incubated with Cy-chrome-labeled CD1a and PE-labeled Langerin after permeabilization. Saponin permeabilization was used for detection of intracellular FcεRI α , whereas digitonin permeabilization was used for detection of FcεRI γ expression. Cells were acquired using a FACSCalibur flow cytometer (BD) as described in detail elsewhere (Novak *et al*, 2001). For quantitative evaluation, the CD1a⁺ population was gated manually and the percentage of FcεRI α ⁺ or FcεRI γ ⁺ cells was determined using Lysis II software (BD).

Immunolabeling for phenotyping was performed as reported (Wollenberg *et al*, 1999). As a control, cells were stained with corresponding isotype-matched control immunoglobulin. All incubations and washes were performed at 4°C. Results are expressed as percent of positive cells.

TGF- β 1 levels in cord blood plasma and culture supernatants were determined by ELISA according to the manufacturer's protocol (Quantikine, R&D Systems) and as described in detail (Novak *et al*, 2001).

Amplification of mRNA and analysis of transcripts Total RNA was extracted after 9 d of culture from highly purified CD34-derived CD1a⁺ DC using Trizol (Life Technologies, Eggenstein, Germany) following the manufacturer's instructions. RT reactions were performed using 1 μ g of total RNA. Denaturation at 94°C for 40 s was followed by annealing of the primers at 55°C for 30s and extension at 72°C for 30s. A final extension phase of 5 min was added. Specific primer sequences for each gene were as follows: human β -actin: sense, 5'-GAG CGG GAA ATC GTG CGT GAC ATT-3'; antisense, 5'-GAT GGA GTT GAA GGT AGT TTC GTG-3', yielding a fragment of 240 bp; human FcεRI γ : sense, 5'-CTG TTC TTC GCT CCA GAT GGC GT-3'; antisense, 5'-TAC AGT AAT GTT GAG GGG CTC AG-3' (fragment of 536 bp), human FcεRI γ sense, 5'-CCA GCA GTG GTC TTG CTC TTA C-3' and antisense: 5'-GCA TGC AGG CAT ATG TGA TGC C-3' (fragment of 338bp), human FcεRI β sense, 5'-GGA CAC AGA AAG TAA TAG GAG AG and antisense: 5'-GAT CAG GAT GGT AAT TCC CGT T (fragment of 446 bp) and human tryptase sense, 5'-CTC CCT CAT CCA CCC CCA GT and antisense: 5'-GGA TCC AGT CCA AGT AGT AG (fragment of 616 bp). Amplification was performed on a Perkin-Elmer Gene Amp PCR System 9600 thermocycler (Applied Biosystems, Weiterstadt, FRG). The PCR cycle numbers for the amplification of the respective cDNAs were 25 for β -actin and 30 for FcεRI α and FcεRI γ . Specific PCR fragments were separated on a 1% agarose gel and visualized using ethidium bromide staining. The PCR products were evaluated semiquantitatively by comparing the ratio of the specific products versus the β -actin band by digital image analysis using the WinCam system (Cybertech, Berlin, Germany).

T cell proliferation assays Proliferation assays were performed in a total volume of 200 μ L in 96-well round-bottom plastic culture plates using allogeneic T cells as responder cells. Allogeneic T cells were isolated from PBMC of healthy volunteers using a nylon-wool column (> 85% purity as assessed by anti-CD3 staining). DC were cultured as described above until day 9 of culture. In allogeneic assays, triplicates of DC containing 100, 200, or 1000 viable CD1a⁺ DC/well were incubated with 100,000 viable allogeneic T cells at 37°C for 3 d. Proliferative response was then measured by addition of 1 μ Ci 3H-thymidine incorporation for 12 h. The incorporated radioactivity was measured in a liquid scintillation counter (1450 MicroBeta Trilux, Wallac). Relative stimulation indices (rSI)

were calculated as follows: $rSI = (c.p.m. (DC + T \text{ cells}) - c.p.m. (T \text{ cells}) / cpm (T \text{ cells})$.

Statistical analysis For statistical evaluation of significances, the Wilcoxon test was performed. Correlations were calculated by Pearson's linear regression analysis. These tests were realized using the SPSS 10.0 software (SPSS, Chicago, Illinois). Results are shown as arithmetic mean \pm standard error of the mean (SEM). *, $p < 0.05$, **, $p < 0.01$, n.s., not significant.

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