Impact of Antigen-unloaded Immature Dendritic Cells on Antileukemic T-Cell Cytotoxicity

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Abstract. *Background: Immature dendritic cells (iDC) loaded with antigens are able to induce tolerance in antigen-specific T-cells. The potential of antigen-unloaded iDC to regulate the antileukemic cytotoxicity of autologous T-cells was determined. Materials and Methods: iDC generated with 50 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and very immature DC (viDC) generated with 10 U/ml GM-CSF from the bone marrow of Balb/c mice were used for T-cell co-culture. Results: The measurement of cellular cytotoxicity against the syngeneic murine B-cell leukemia line A20 revealed that T-cells without co-culture or after co-culture with iDC exerted a similar cytotoxicity, whereas T-cells co-incubated with viDC showed a significantly diminished lysis of A20 cells ($p<0.05$). Conclusion: Antigen-unloaded iDC in contrast to antigen-loaded iDC may not affect antileukemic T-cell cytotoxicity, whereas antigen-unloaded DC cultures generated with a low dose of GM-CSF are able to impair the T-cell-mediated cytolysis of leukemic cells.*

Dendritic cells (DCs) are professional antigen-presenting cells (APC) which possess a unique regulatory function on T-cell immunity dependent on their maturation stage (1-3). Several studies have shown that phenotypically immature DCs (iDCs) due to low expression of costimulatory and major histocompatibility complex (MHC) class II-molecules are weak stimulators of naive allogeneic T-cells and can instead induce alloantigen-specific T-cell hyporesponsiveness (4-6). Moreover, iDCs loaded with tumor- or virus-derived antigen efficiently down-regulated antigen specific T-cell activity (7, 8). Furthermore, in the autologous system, iDCs have been shown to be involved in the induction and maintenance of T-cell tolerance against self-antigens in peripheral tissues (9-11). iDCs generated with a low dose of granulocyte-macrophage colony-stimulating factor (GM-CSF) displayed, in addition to their tolerogenic potential, a stable phenotype resistant to inflammatory maturation stimuli such as lipopolysaccharide (LPS) or TNF-α (12). The tolerogenic effect of antigen loaded iDCs on naïve or antigen experienced T-cells is mediated either directly by insufficient costimulation and low secretion of T-cell activating cytokines such as interleukin (IL)-12 leading to T-cell anergy or by the induction of immunosuppressive regulatory T (Treg) cells (4, 7, 8, 13, 14). However, in contrast to the well-described antigen-specific inhibition of effector T-cell functions by antigen loaded iDC, only a few reports have mentioned a potential regulatory effect of antigen-unloaded DCs on naive or preactivated T-cells (7, 8, 15). This might be of interest since iDCs residing in many peripheral tissues are initially free from captured antigen apart from the presentation of self-antigens. To analyze a potential role of antigen-unloaded iDCs on the antileukemic cytotoxicity of autologous T-cells *in vitro* the syngeneic murine B-cell leukemia/lymphoma cell line A20 (Balb/c) was chosen as a T-cell target. The A20 tumor is capable of stimulating autologous cytotoxic T lymphocytes (CTL) with low to moderate cytotoxicity due to its constitutive expression of costimulatory molecules and significant amounts of MHC class-II molecules (16-18). In this study, antigen unloaded iDC generated with different concentrations of GM-CSF were compared for their regulatory potential on the specific T-cell cytotoxicity to A20 lymphoblastic cells.

Materials and Methods

Cell line and culture. The B-lymphoblastic leukemia/lymphoma line A20 was kindly provided by Genethor GmbH, Berlin, Germany, and was cultured in RPMI-1640 medium supplemented with 10% FCS, 50 μg/ml gentamicin (all from Biochrom, Berlin, Germany) and 10 μg/ml β-mercaptoethanol (Sigma, Taufkirchen, Germany).

Generation of DCs. Bone marrow cells were flushed with PBS (Biochrom) from the femurs and tibias of 8- to 12-week-old Balb/c mice (H-2^d, Charles River, Sulzfeld, Germany). The cells were

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cultured in 6-well tissue culture plates (Falcon, Becton Dickinson GmbH, Heidelberg, Germany) at an initial density of 1×10^6 in 4 ml/well RPMI-1640 medium supplemented with 10% heat-inactivated FCS and 50 μg/ml gentamicin (Biochrom, Berlin, Germany). For the generation of very immature DCs (viDCs) and iDCs, 10 or 50 U/ml of mouse GM-CSF (Peprotech/Tebu, Frankfurt, Germany) were added, respectively. At day three of culture, the plate was swirled gently and half of the medium was replaced with 3 ml of fresh complete medium containing the appropriate concentration of GM-CSF. At day five 2 ml of the medium was replaced by the same volume of fresh complete medium. At day seven or eight the cells were used for experiments.

Immunophenotyping of DCs and A20 cells. To determine the phenotype of the DCs, the A20 cell line and after incubation with irradiated tumor cells of the splenic lymphocytes (Balb/c), the following fluoroisothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-mouse monoclonal antibodies (mAbs) were used: CD11c (N418), MHC-class II (AMS.32.1), CD80 (16-10A), CD86 (GL1) (Becton Dickinson, San Jose, CA, USA), CD4 (GK1.5), CD8α (53-6.7) (Natutec, Frankfurt, Germany), B220 (RA3-6B2) (Caltag, San Francisco, CA, USA), and the relevant isotype control antibodies (Pharmingen, San Diego, CA, USA). Binding of the biotin-conjugated anti-mouse NK-1.1 mAb (PK136) (Pharmingen) was detected by subsequent staining with *Rhodophyta* phycoerythrin (R-PE)-coupled streptavidin molecules (Jackson Immunoresearch, West Grove, PA, USA). Samples of 1×10^5 cells were stained with the respective antibodies for 30 min on ice in PBS containing 2% FCS and 0.1% sodium azide and then measured with a FACScan (Becton Dickinson, Heidelberg, Germany), and the data were analyzed using CellQuest software (Becton Dickinson).

Generation of T effector cells. Balb/c-Mice were narcotized with ether and sacrificed before the spleen was removed. Single cell suspensions from the spleens were prepared by gently pressing the tissue through a 70 μm cell strainer (Falcon; Beckton Dickinson) using a syringe plunger to remove connective tissue and debris. Erythrocyte lysis was performed by the addition of an equal volume of distilled water to the cells suspended in 1 ml PBS and incubation for 30 s. Subsequently, the cells were washed twice with RPMI-1640 medium/10% FCS before they were used for the experiments. Splenocytes (1×10^6) were incubated with 0.5×10^5 γ-irradiated A20 cells (45-60 GY) over 7 days in 24-well plates in RPMI-1640 medium supplemented with 10% FCS, 50 μg/ml gentamicin, 10 U/ml IL-2 (Proleukin, Chiron, Ratingen, Germany) and 10 μg/ml β-mercaptoethanol. The strong formation of rosettes was observed between the A20 cells and the T-cells. Subsequently, the *in vitro* activated splenic lymphocytes (1×10^5) containing 52±4% $CD4^+$ T-cells, 9±3% $CD8^+$ T-cells, 25±2% B cells and 4±1% NK cells (data from two independent determinations) were incubated with the DCs (1×10^6) over four to five days in 24 well plates in 1 ml RPMI 1640 medium supplemented with 10% FCS and gentamicin (50 μg/ml) or were rested in medium with 5 U/ml IL-2. After DC-T-cell co-culture, the cells were harvested, washed and used as effector cells.

Cytotoxicity assay. T-cell cytotoxicity to A20 leukemia target cells was determined by a flow cytometric CTL-assay using fluorescent dyes for the specific staining of target and effector cells as described in related CTL or cell death assays (19-22). The lipophilic 5- and 6- carboxyfluorescein diacetate succinimidylester (CFDA-SE) (Sigma) was used for the labeling of target cells and the DNA stain propidium iodide (PI, Sigma) served for the distinction of live and dead target cells. In brief, 1×10^6 target cells were stained with 0.5 μM CFDA-SE in PBS. After uptake the non-fluorescent dye is cleaved by esterases into the green fluorescent carboxyfluorescein succinimidylester (CFSE) and irreversibly binds to intracellular proteins (16). Effector cells (2.5×10^5) were incubated with 5×10^3 target cells in triplicate for 5 h in a 96-well V-bottom microtiter plate in 150 μl RPMI-1640, 10% FCS and 50 μg/ml gentamicin at 37°C and 5% CO_2. The CTL activity was stopped by placing the probes on ice and 2 μg/ml PI were added immediately before measuring control samples without effector cells and 5×10^4 cells of the samples containing target and effector cells using a FACScan (Becton Dickinson). Target cell lysis was determined by the separation of effector cells from the CFDA-SE labeled target cells and subsequently, by applying histogram statistics to define the percentage of PI+ (dead) target cells. Cytolysis was corrected by the percentage of dead cells in the control samples, representing the unspecific background cell death. The analysis of flow cytometric data was performed with the CellQuest software (Becton Dickinson).

Statistics. Data are presented as mean±SEM. The Student's *t*-test was used to determine significant differences ($p<0.05$) between the cytotoxicity of differently treated T-cells.

Results

Phenotype of the DC cultures and A20 cells. The flow cytometric analysis of surface markers at day seven or eight revealed a low expression of CD80 and CD86 molecules on the $CD11c^+$ viDCs whereas both molecules were moderately expressed on the $CD11c^+$ iDCs (Figure 1). The expression of MHC class II molecules exhibited a markedly lower intensity on the viDCs than on the iDCs. The percentages of $CD11c^+$ cells ranged in the viDC cultures from 30% to 50% and in the iDC cultures from 40% to 70% (data not shown). The A20 cells revealed a relatively mature B cell phenotype due to low to moderate expression of CD80 and CD86 molecules and high expression of MHC class II molecules (data not shown).

Flow cytometric measurement of T-cell cytotoxicity. CFDA-SE staining of the target cells led normally to >90% dye positive cells and enabled gating to specifically separate the target cells from the effector cells (Figure 2A and B). The distinction between vital and dead tumor cells by PI staining demonstrated a low background lysis in the control samples without effector cells (Figure 2C) and low to moderate cytolytic activity of autologous T-cells at an E:T ratio of 50:1 (Figure 2D).

T-cell cytotoxicity regulated by DCs. To determine the effect of antigen unpulsed DCs exhibiting an immature phenotype on the cytotoxicity of tumor-specific activated T-cells

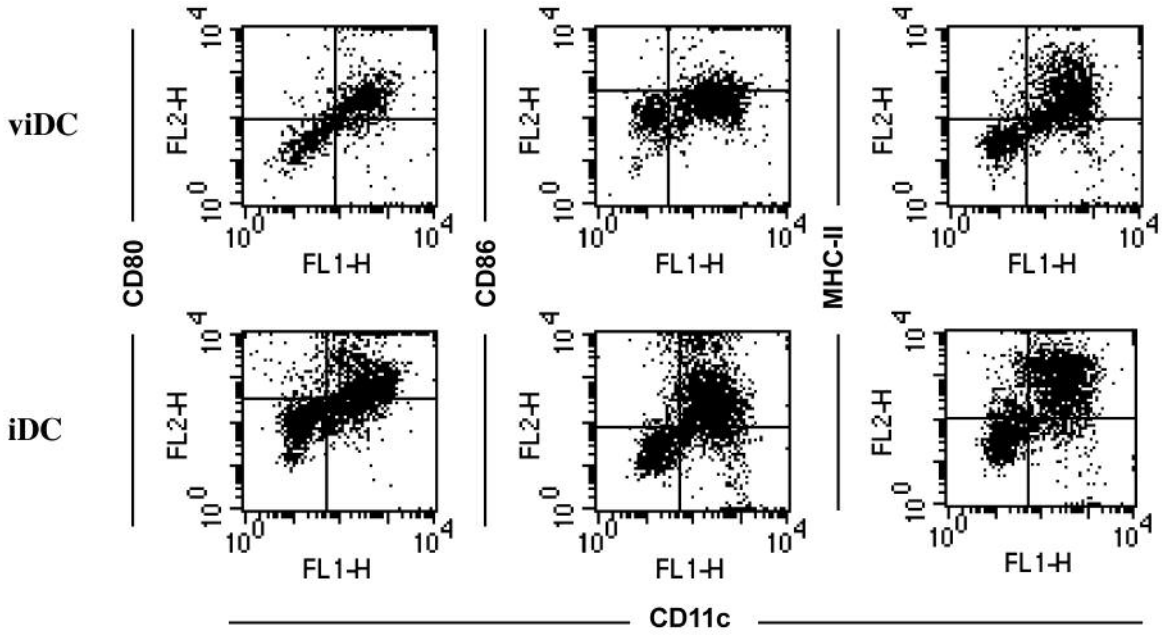

Figure 1. *Expression of co-stimulatory molecules on CD11c$^+$ cells in viDC and iDC cultures at day seven of differentiation. Flow cytometric analysis indicated expression of CD80 and CD86 molecules with low intensity on 84% and 31% of the viDCs, respectively. CD80 and CD86 molecules were expressed on 71% and on 77% of the iDCs with moderate intensity, respectively. MHC class II molecules were found with low to moderate intensity on 81% of the viDCs and with moderate to high intensity on 90% of the iDCs. Data shown are representative of three independent experiments with similar results.*

autologous co-cultures were performed between activated splenic lymphocytes and DCs. T-cells without DC co-culture exerted a moderate target cell lysis after *in vitro* incubation with γ-irradiated syngeneic leukemia cells. The T-cell mediated cytolysis was significantly diminished after co-culture of splenic lymphocytes with viDCs and was maintained or slightly reduced after co-culture with iDCs (Figure 3).

Discussion

In the present study, the T-cell cytotoxicity against syngeneic A20 leukemia cells was in the same range as reported in previous studies and could be exerted by both CD4$^+$ and CD8$^+$ T-cells (18). Due to the expression of significant amounts of MHC class I and II molecules on the leukemic cell line, NK cells should not have contributed significantly to the cytolysis. The viDC cultures showed an immature phenotype similar to DCs generated with low dose-GM-CSF from other studies (4, 12, 23). In contrast, the iDC cultures revealed a higher maturation state than viDCs, partly advanced to the stage of semi-mature DCs (24). The tolerogenic function of immature DC cultures has been attributed to the induction of T-cell anergy or Treg cells, mainly due to insufficient co-stimulation and the release of no or very low amounts of T-cell activating cytokines such as IL-12 (4, 7, 8, 14).

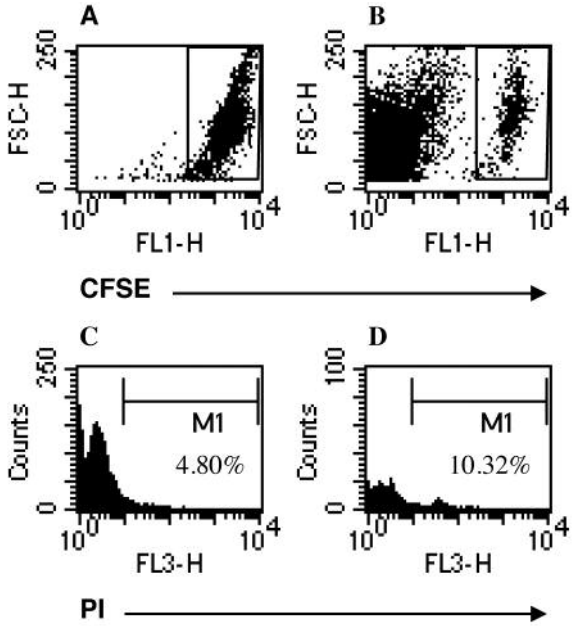

Figure 2. *Flow cytometric assay for the determination of autologous T cell cytotoxicity. CFSE stained target cells (5×10^3) remained untreated as control (A), or were co-incubated with effector cells at an E:T-ratio of 50:1 in 96-well plates for 5h (B). To assess cellular cytotoxicity CFSE$^+$ target cells were gated to exclude unlabeled effector cells and debris. Subsequently, the percentages of PI$^+$ target cells were assessed by histogram statistics (C, D). The cytolysis was corrected by the spontaneous cell death of target cells incubated without effector cells (C). Data show one representative example of several experiments.*

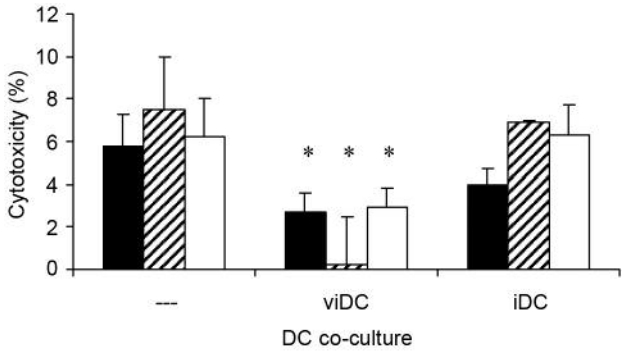

Figure 3. *Regulation of autologous T cell cytotoxicity by the DC phenotype. Splenic lymphocytes prestimulated with γ-irradiated A20 cells were co-cultured with DCs at a ratio of 10:1. Subsequently, the lymphocytes were used in flow cytometric 5 h CTL-assays against A20 cells. The autologous T cells showed significantly decreased cytotoxicity after co-culture with viDC (*p<0.05). Data show three independent experiments each performed in triplicate ± SEM.*

In the present experiments the DCs presented self-antigens, but no tumor antigens to the syngeneic T-cells which were immunologically tolerant to the self-antigens. Therefore, the down-regulation of T-cell cytotoxicity after viDC-T-cell co-culture could result rather from the activation of non-antigen specific Treg cells than from the induction of tumor or self antigen-specific MHC class I or II-restricted T-cell anergy (5, 7, 13, 14). In contrast, peptide-loaded IL-10-treated iDCs induced anergy in peptide-antigen-specific syngeneic $CD8^+$ CTL, leading to inhibited proliferation and a comparable failure to lyse an antigen-overexpressing tumor cell line (25).

The lack of a tolerogenic effect of antigen unloaded iDCs on a pre-existing effector T-cell function was in contrast to studies employing antigen loaded or allogeneic iDCs (5-8). The induction of Treg cells or T-cell anergy by the iDCs, leading to decreased T-cell cytotoxicity, could have failed due to their less immature phenotype in comparison to the viDC. In some reports the potential of iDCs to inhibit T-cell effector functions was limited and largely restricted to resting or naïve T-cells; for example the induction of murine IL-10 producing $CD4^+$ Treg cells required repetitive stimulation of naïve T-cells by allogeneic iDCs (5). Human iDCs have been converted into strongly tolerogenic DCs by treatment with IL-10 during their generation (25-28).

However, besides a potential role of $CD11^+$ DC in the regulation of T-cell effector function, the tolerogenic effect of viDC cultures could rely in part on the suppressive activity of recently identified granulocyte-differentiation antigen 1 $(Gr\text{-}1)^+$, $CD11b^+$ and $CD11c^-$ myeloid suppressor cells (MSC) (29). The myeloid DC precursors appear transiently as non-adherent cells in murine bone marrow cultures 8-10 days after treatment with a similar low dose of GM-CSF as used for the generation of viDCs. These cells inhibited allogeneic and ovalbumin-specific $CD4^+$ and $CD8^+$ T-cell responses *via* cell contact and nitric oxide production *in vitro* and their depletion restored antigen specific $CD8^+$ CTL activity allocating MSC a key role in the regulation of T-cell effector functions (30, 31).

In summary, evidence is presented that viDC cultures are able to down-regulate tumor-specific T-cell cytotoxicity, although lacking the presentation of tumor-derived antigens.

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