Ph.D. Thesis

Tolerogenic Dendritic Cells:
Immunomodulation of Monocyte-Derived Dendritic Cells with
n-Butyrate, NFκB Inhibitor PDTC and JAK3 Inhibitor WHI-P-154

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Using the cell culture model of monocyte-derived DC, we generated and described a novel form of tolerogenic DC by activating immature DC in the presence of NFκB inhibition with PDTC. Importantly, the successful establishment of alloantigenic hyporesponsiveness is not prevented by concomitant calcineurin inhibition in vitro as well as in T cells from patients under Cyclosporine A-based immunosuppression ex vivo. Data obtained from the present study indicate that the potential benefit of DC-based protocols as a therapeutic strategy will not be lost if this treatment modality is incorporated into conventional clinical immunosuppressive treatment. The JAK3 inhibition with the rationally designed JAK3 inhibitor WHI-P-154 arrested the CD40-triggered DC at an immature level. These results suggest that immunosuppressive therapies targeting the tyrosine kinase JAK3 may also affect the function of myeloid cells. This property of JAK3 inhibitors therefore represents a further level of interference, which together with the well-established suppression of common-gamma chain signaling could be responsible for their clinical efficacy.

We demonstrate that T-cell stimulation by TCR triggering in the presence of a pharmacologic JAK3 inhibitor is characterized by defective early signaling events leading to a profound impairment of regular T-cell activation. Therefore the selective prevention of JAK3 activity in peripheral T cells may have important implications for future immunosuppressive therapy.

CONCLUSIONS

In conclusion, given the therapeutical potential of dendritic cells to induce tolerance in general, these findings suggest that immunization strategies using in vitro modulated dendritic cells that are characterized by a maturation blockade might be potentially useful in the control of allograft rejection.
4.) Effect of JAK3 Inhibitor Treatment on Homotypic T-Cell Aggregation

T cells activated with CD3 and CD28 mAbs exhibited prominent cell clusters 8 hr after initiation of the culture, JAK3 inhibitor-supplemented cultures were completely devoid of any T-cell clusters.

SUMMARY OF THE RESULTS

These modulated DC exhibited phenotypical features and cytokine production of immature DC, as well as defective stimulatory capacity for allogeneic T-cell responses. Up-regulation of the costimulatory molecules CD80, CD86 and CD40, the MHC antigens as well as of the specific DC maturation marker CD83 was found to be clearly suppressed. Endocytosis and macropinocytosis were inhibited. We observed a clear inhibition of the production of the immunostimulatory cytokine IL-12. We found reduced T-cell stimulatory capacity of DC. These modulated-DC induced a state of hyporeactivity in alloreactive T cells in secondary cultures. Addition of CsA to primary MLC led to an inhibition of the allogeneic T-cell proliferation. Secondary MLC revealed that the presence of CsA during alloantigenic priming did not influence the induction of the hyporesponsive state by PDTC-modulated DC.

DISCUSSION

We provide phenotypical, morphological, and functional evidence that the physiologically occurring bacterial metabolite n-butyrate profoundly suppresses the development and maturation of monocyte-derived DC in vitro resulting in impaired DC function. Findings, concerning the n-butyrate pretreatment of DC, beyond its anti-inflammatory properties suggest a possible in vivo immunomodulatory role of n-butyrate via interference with the function of DC in the gastrointestinal tract.

INTRODUCTION

Dendritic cells (DC) are professional antigen-presenting cells (APC), which are the most potent inducers of primary T-cell responses. They are uniquely specialized among all leukocytes to couple the presentation of antigen, bound to major histocompatibility complex (MHC) molecules, with all the adhesive and costimulatory signals, collectively termed accessory molecules, required to initiate cellular immune responses. Beyond this immunostimulatory function, certain DC subsets have been shown to induce T-cell tolerance in vitro and in vivo. Various attempts have been made to convert DC into tolerogenic antigen presenting cells (APC). These treatment modalities have in common the ability to interfere with the proper maturation of DC. A major step towards the realization of allograft tolerance may represent the generation of tolerogenic DC facilitating graft acceptance and maintenance of T-cell hyporeactivity.

AIMS OF THE STUDY

Our aim was to generate tolerogenic monocyte-derived human dendritic cells in vitro by activating immature DC in the presence of different inhibitory substances. In this study three independent attempts were made to convert DC into tolerogenic APC such as treatment of DC with (A) n-butyrate, (B) NFκB inhibitor pyrrolidine dithiocarbamate (PDTC) and (C) Janus Kinase 3 inhibitor WHI-P-154. We aimed to characterize these in vitro modulated-DC and to analyze the hyporesponsiveness induced by these APC. Furthermore, we aimed to study the influence of calcineurin inhibition with Cyclosporine A (CsA) in functional T-cell responses elicited by DC pretreatment with PDTC in primary and secondary mixed lymphocyte culture (MLC) and to establish T-cell hyporesponsiveness with PDTC-modulated donor dendritic cells in allogeneic T cells from patients with renal allografts under CsA-based immunosuppression.
In the current work we aimed to study the followings:

A.) “Bacterial Metabolite Interference with Maturation of Human Monocyte-Derived Dendritic Cells”

1.) To investigate how monocyte-derived dendritic cells respond to the bacterial stimulus (lipopolysaccharide) when applied in the presence of n-butyrate with regard to phenotypical and morphological changes, T-cell stimulatory capacity and analysis of the DC antigen-uptake machinery.

B.) “Hyporesponsiveness in Alloreactive T-Cells by NFκB Inhibitor-Treated Dendritic Cells: Resistance to Calcineurin Inhibition”

1.) To generate tolerogenic monocyte-derived dendritic cells by activating immature DC in the presence of NFκB inhibitor pyrrolidine dithiocarbamate (PDTC) in order to obtain DC that exhibit phenotypical features and cytokine production of immature DC.
2.) To characterize allostimulatory potential of PDTC-modulated dendritic cells in primary MLC, as well as the expression of activation markers and cytokine production of allogeneic T-cells.
3.) To induce allogeneic hyporesponsiveness by PDTC-modulated dendritic cells and to analyze this state of allogeneic tolerance after restimulation in secondary MLC.
4.) To study the influence of calcineurin inhibition with Cyclosporine A (CsA) in functional T-cell responses elicited by different DC pretreatment protocols with PDTC in primary and secondary MLC.
5.) To establish T-cell hyporesponsiveness with PDTC-modulated donor dendritic cells in allogeneic T cells from patients with renal allografts under CsA-based immunosuppression.

C.) “Prevention of CD40-Triggered Dendritic Cell Maturation and Induction of T-Cell Hyporeactivity by Targeting of Janus Kinase 3”

1.) Targeting JAK3 disrupts CD40-triggered DC maturation

Up-regulation of the costimulatory molecules CD80, CD86 and CD40, the MHC class I and II molecules as well as of the specific DC maturation marker CD83 was inhibited. JAK3 inhibitor-treated DC did not revert to a monocyte/macrophage stage.

2.) JAK3 inhibition does not interfere with the activation-associated down-regulation of the DC-antigen uptake machinery

Endocytosis and macropinocytosis, which were evaluated by incorporation of FITC-labeled dextran or of lucifer yellow (LY), respectively, were more pronounced in immature DC than in CD40-activated DC. Interestingly, endocytosis and macropinocytosis was similar in JAK3 inhibitor-treated DC and mature DC. Furthermore, CD40 ligation led to a reduction in mannose receptor expression in DC. As observed for antigen uptake, JAK3 inhibitor-treated DC behaved similar to mature DC with regard to mannose receptor expression.

3.) Prevention of T-cell stimulatory capacity and induction of antigen-specific hyporeactivity by JAK3 inhibited DC

CD40 triggering of DC induced a vigorous T-cell stimulating capacity compared with immature DC. Treatment of DC with the JAK3-inhibitor during CD40 triggering resulted in an allostimulatory capacity similar to immature DC. Pre culturing allogeneic T cells with CD40-triggered DC led to a strong secondary T-cell alloresponse. In contrast, preculture with CD40-triggered DC exposed to the JAK3 inhibitor resulted in a dramatically reduced responsiveness of allogeneic T cells upon restimulation. Restimulation with allogeneic mature DC from unrelated third-party donors was also associated with defective T-cell proliferation in JAK3-treated DC or immature DC-primed T cells, indicating nonspecific T-cell hyporesponsiveness induced by both APC populations. Addition of IL-2 led to a complete restoration of the defective T-cell proliferation of T cells cocultivated with JAK3 inhibitor-pretreated DC in primary MLC.
3.) Induction of allogeneic hyporesponsiveness by PDTC-modulated DC

T cells exposed to PDTC-modulated DC did not proliferate upon challenge with immature or even fully mature DC from both original and third party donor DC. In contrast, APC-independent T-cell stimulation with PMA plus OKT-3 was unaffected. Recombinants human IL-2 partially restored proliferative responses towards allogeneic DC while it induced an unspecified increase in secondary proliferative responses. In the absence of T-cell receptor (TCR)-mediated activation, IL-2 alone induced a low grade of proliferation in tolerant T cells. Hyporesponsive T cells were unable to significantly suppress allogeneic activation of syngeneic T cells and allowed T-cell responsiveness comparable to T cells previously exposed to immature DC or immature DC treated with PDTC. PDTC-modulated DC were unable to suppress alloreponsiveness to mature DC from the same donor.

4.) CsA does not counteract the suppressive state induced by PDTC-modulated dendritic cells

Addition of CsA to primary MLC led to an inhibition of the allogeneic T-cell proliferation regardless of the mode of APC pretreatment. The presence of CsA had no influence on the proliferative response of T cells in secondary cultures when they were stimulated with immature or mature DC in primary MLC. Interestingly, secondary MLC revealed that the presence of CsA during alloantigenic priming did not influence the induction of the hyporesponsive state by PDTC-modulated DC.

5.) T-cell hyporesponsiveness with PDTC-modulated DC in T cells from patients with renal allografts under calcineurin inhibitor-based immunosuppression

While the primary MLC response was profoundly reduced using PDTC-modulated DC, a conspicuous state of T-cell hyporesponsiveness was observed in restimulation cultures supplemented with allogeneic stimulator cells. In contrast, T-cell responsiveness was unimpaired when various polyclonal stimuli were employed. It is concluded that induction of T-cell hyporeactivity towards alloantigens by maturation-resistant DC is not dependent on calcineurin activation and furthermore is feasible in T cells exposed to CsA in vivo.

C.) “Prevention of CD40-Triggered Dendritic Cell Maturation and Induction of T-Cell Hyporeactivity by Targeting of Janus Kinase 3” 2,3,5

1.) To explore the impact of Janus Kinase 3 (JAK3) inhibition with WHI-P-154 (4-(3'-Bromo-4'-hydroxyphenyl) amino)-6,7-dimethoxyquinazoline) on the phenotype of monocyte-derived dendritic cells activated through CD40 engagement.

2.) To evaluate receptor-driven endocytosis, macropinocytosis and mannose receptor expression of JAK3 inhibitor-treated dendritic cells.

3.) To investigate T-cell stimulatory potential of JAK3 inhibitor-treated dendritic cells in primary MLC, to induce hyporesponsiveness after restimulation in secondary MLC and to assess the specificity and reversibility of this hyporesponsive state.

4.) To evaluate homotypical cell clustering of T cells activated with CD3 and CD28 mAbs in the presence of Janus Kinase 3 inhibitor WHI-P-154.

PATIENTS AND METHODS

Patients

Patients included in the present study had received a cadaveric kidney transplant at least 2 years prior to evaluation of T-cell function. All patients had stable long-term allograft function and were on Cyclosporin A-based triple immunosuppression.

Cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of blood donors by density gradient centrifugation over Lymphoprep. For monocyte enrichment, PBMC were depleted of T cells by sheep erythrocyte-rosetting. Resting T cells were isolated by magnetic selection. For DC generation from cadaveric kidney donors, the spleen cells were isolated by density gradient centrifugation. The mononuclear population was then used for DC differentiation.
Monocytes from healthy donor or mononuclear cells from cadaveric spleens were cultured in 24-well plates at a cell density of $5 \times 10^5$ cells/ml in RPMI-1640 / 10% FCS medium. For induction of differentiation, cells were cultured for 5 d with rh-GM-CSF and rh-IL-4. To induce final maturation LPS or CD40L followed by cross-linking with anti-FLAG mAbs was then added for 48 h. The inhibitors were added 2-48 h before activation with the respective DC maturation stimuli.

**Fluorescein-activated cell sorter (FACS) and cytokine production**

For evaluation of surface-marker (MHC I and II, CD40, CD80, CD86, CD83) expression analyses were performed by FACS. Mannose receptor-mediated endocytosis was determined with FITC-labeled dextran. Fluid-phase endocytosis was measured via cellular uptake of lucifer yellow. Supernatants were harvested and the cytokines were measured by sandwich ELISA.

**Allogeneic mixed lymphocyte culture (MLC) and tolerance assays**

Stimulator cells were irradiated and added to allogeneic T cells in 96-well culture plates in RPMI-1640/10% FCS medium. After 4 days $[^3]H$-thymidine incorporation was utilized to assess T-cell proliferation. After another 18 h, the cells were harvested and radioactivity was determined usilicate cultures. For secondary MLC a microplate scintillation counter. DNA synthesis was expressed as mean cpm of triplicate cultures, purified T cells or PBMC (from renal allografts recipients) were mixed with DC subjected to the various treatment protocols. After washing at day 7, the cells were re-plated with irradiated cells from the original donor or unrelated third party donors. In selected experiments, secondary cultures were carried out in the presence of rhIL-2. Global T-cell reactivity in secondary culture was assayed by stimulation with PMA and OKT-3.

**Statistics**

Comparisons were performed by using the paired Student’s t-test. A p-value of $< 0.05$ was considered statistically significant.

**RESULTS**

A.) “Bacterial Metabolite Interference with Maturation of Human Monocyte-Derived Dendritic Cells”

1.) Phenotypical and functional impairment of DC maturation by n-butyrate

Expression of CD25 and CD83 and of critical costimulatory molecules, i.e., CD40, CD80, and CD86, was reduced substantially. A clear suppression of the up-regulation of MHC class I and II antigens was observed. Little or no cell clustering occurred in cultures, moreover, the majority of cells imposted with widespread cytoplasmic projections. We found a significant dose-dependent reduction of the allostimulatory capacity. The expression of the antigen-uptake molecules CD32 and mannose receptor was inhibited. Assessment of macropinocytosis revealed impaired internalization of lucifer yellow but a less pronounced inhibition of receptor-mediated endocytosis of dextran molecules.

B.) “Hyporesponsiveness in Alloreactive T-Cells by NFκB Inhibitor-Treated Dendritic Cells: Resistance to Calcineurin Inhibition”

1.) Arrest in DC maturation by treatment with the NFκB inhibitor PDTC

Expression of CD83 and of critical costimulatory molecules such as CD40, CD80 and CD86 was reduced substantially. Up-regulation of MHC class II antigens was found to be clearly suppressed. We observed a clear inhibition of the production of the immunostimulatory cytokines IL-12 and TNF-α.

2.) PDTC-modulated DC exhibit defective stimulatory capacity for allogeneic T-cell responses

Pretreatment with PDTC induced a DC population with a low stimulatory capacity. T-cells challenged with modified DC did not express CD69, similarly expression of CD25 was profoundly impaired. We found a dose-dependent suppression of IL-2 and IFN-γ production.