Friend retrovirus infection of myeloid dendritic cells impairs maturation, prolongs contact to naïve T cells, and favors expansion of regulatory T cells

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Abstract

Retroviruses have developed immunomodulatory mechanisms to avoid being attacked by the immune system. The mechanisms of this retrovirus-associated immune suppression are far from clarified. Dendritic cells (DC) have been attributed a decisive role in these pathogenic processes. We have used the Friend retrovirus (FV) mouse model in order to acquire further knowledge about the role of infection of DC in virus-induced immunosuppression.

About 20% of the myeloid DC that were generated from the bone marrow of FV-infected mice carried FV proteins. The infection was productive, and infected DC transmitted the virus in cell culture and in vivo. FV infection of DC led to a defect in DC maturation as infected cells expressed very little co-stimulatory molecules. Live imaging analysis of the cell contact between DC and T cells revealed prolonged contacts of T cells with infected DC as compared to uninfected DC. Although naïve T cells were still activated by FV-infected DC this activation did not result in antigen-specific T cell proliferation. Interestingly, infected DC expanded a population of Foxp3-positive regulatory T cells with immunosuppressive potential suggesting that the contact between naïve T cells and retrovirus-infected DC results in tolerance rather than immunity. Thus, retroviral infection of DC leads to an expansion of regulatory T cells, which might serve as an immune escape mechanism of the virus.
Introduction

Dendritic cells (DC) are the most potent antigen presenting cells of the immune system and they are pivotal in the initiation of immune responses against viruses. However, a number of viruses are able to infect DC and several recent studies have investigated the effect of such infections on the biology of DC. While only very few studies report enhanced or unchanged functions of DC after viral infection, the majority of viruses seem to impair functional properties of DC (for review see 2). These data imply that viruses infect DC as a strategy of immune escape. In most reports that demonstrate the impairment of DC by viruses, the morphology, phenotype, viability, and the ability to secrete cytokines of infected DC was found to be altered, suggesting that viruses might have a significant impact on the DC-mediated initiation of an immune response in an infected host. In fact, there is one report that directly shows that measles virus infection of DC can result in unstable DC – T cell contacts and as a consequence in impaired T cell activation. Retroviruses, like human immunodeficiency virus, are also known to infect DC and interfere with their maturation. Maturation of DC can easily be measured by analyzing expression of co-stimulatory molecules, like CD40, CD80, and CD86, or maturation markers like the molecule CD83. However, the biological consequences of retrovirus infection of DC for antigen presentation to T cells has not been investigated in detail so far. The most important functional properties of DC are T cell engagement and subsequent activation, which is the critical step in inducing adaptive immunity after infection. Since the interaction of DC and naïve T cells requires physical cell-cell contact, we used a three-dimensional collagen matrix model to investigate the contact duration and kinetics of virus-infected DC with naïve T cells. FV is a retroviral complex comprised of two components: a replication-competent helper virus called Friend Murine Leukemia virus (F-MuLV) which is non-pathogenic in adult mice; and a
replication-defective but pathogenic component called spleen focus forming virus (SFFV). Co-infection of cells by the two viruses allows SFFV to spread by being packaged into F-MuLV-encoded virus particles. FV infection of susceptible adult mice induces polyclonal proliferation of erythroid precursor cells causing severe splenomegaly. This proliferation is caused by the binding of the SFFV envelope glycoprotein to the erythropoietin receptors of nucleated erythroid cells. In susceptible mice, FV subsequently transforms erythroid precursor cells leading to fully malignant erythroleukemias. However, beside erythroid precursor cells, FV can also infect a variety of other cells types including B cells, monocytes and granulocytes. In addition, FV induces a severe generalized immunosuppression during acute infection, however it is unknown whether this is the result of a functional impairment of antigen presenting cells due to virus infection. Susceptibility to both FV-induced immunosuppression and erythroleukemia is strongly influenced by the genetic background of a given mouse strain. However, resistance to immunosuppression does not directly correlate with recovery from splenomegaly, since some mouse strains are resistant to immunosuppression but still die from erythroleukemia.

Here we show that FV productively infects myeloid DC in vivo and in vitro and interferes with the maturation of these cells. Functional studies indicated that infected DC had prolonged contacts with naïve T cells during antigen presentation but were able to induce antigen-specific T cell proliferation poorly. In contrast, T cells that emerged from co-culture with antigen presenting FV-infected DC expressed markers of regulatory T cells (Treg) and suppress the proliferation of T cells in vitro. Thus, virus-induced alteration of DC is a likely cause of the immunosuppression found in FV infection.
Material and Methods

Mice

BALB/c mice were obtained from Harlan Winkelman laboratories (Borchen, Germany). DO11.10 mice on BALB/c background expressing a transgenic T-cell receptor (TCR), which recognizes ovalbumin peptide 323-339 (ISQAVHAAHAEINEAGR), in the context of I-A\textsuperscript{d}, were from Dr. Blankenstein (Max-Delbrück-Centrum, Berlin, Germany). All mice were housed under specific pathogen-free conditions according to the guidelines of the regional animal care committee and used at 8-12 weeks of age.

Generation and culture of myeloid DC from bone marrow cells

Bone marrow (bm) DC were generated as described by Inaba et al.\textsuperscript{12} with some modifications. In brief, bone marrow cells were collected from murine tibias and femurs, suspended and 3x10\textsuperscript{6} cells were placed in 6-well plate (BD Pharmingen, Germany) containing 5 ml DC Media (RPMI supplemented with 5\% FCS, 1x nonessential amino acids, 2mM L-glutamine, 500nM 2-ME, 100U/ml penicillin/streptomycin, 20\mu g/ml gentamycin), 5ng/ml GM-CSF (R&D Systems, Germany) and 1ng/ml IL-4 (BD Pharmingen, Germany). On day 6, 2x10\textsuperscript{6} non-adherent cells were transferred into a new 6-well plate containing 3ml DC media/well. After a total of 7-9 days of culture, DC were harvested and used in subsequent experiments. For co-culture experiments with T cells DC were stimulated on day 7 for 48h with anti-CD40 (clone: 1C10, a kind gift from S. Amigorena, Paris, France).

Detection of Friend virus-infected DC
To detect FV-infected cells either bone marrow cells or cultured bmDC from FV-infected mice were stained with tissue culture supernatant containing monoclonal antibody (MAb) 34, which is specific for F-MuLV glycosylated Gag protein that is expressed on the surface of infected cells. MAb 34 binding was detected with a goat anti-mouse IgG2b-PE antiserum (BD Pharmingen, Heidelberg, Germany).

**Infectious center**

For infectious center assays infected DC were cocultivated with *Mus dunni* cells at 10-fold dilutions. Cultures were incubated for 5 days, fixed with ethanol, stained with F-MuLV envelope-specific monoclonal antibody 720, and developed with peroxidase-conjugated goat anti-mouse and aminoethylcarbazol to detect foci.

**Electron microscopy**

DC were pelleted by centrifugation and cell pellets were fixed in glutaraldehyde and embedded in 2% agarose. After fixation in osmiumtetroxide these cell blocks were dehydrated in graded ethanols and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and evaluated on a Zeiss EM 902A transmission electron microscope (Zeiss, Oberkochen, Germany).

**Enrichment of FV-infected DC**

For most experiments FV-infected DC were enriched from bmDC cultures. Two different techniques were used to generate DC cultures in which more than 90% of the cells were FV-infected.
1. Bone marrow cells from FV-infected mice were isolated on day 11 post infection as described above and stained with anti MAb 34 and PE conjugated secondary antibody. Infected cells were then isolated using anti-PE microbeads and MACS sorting (Milteny, Bergisch-Gladbach Germany). Cells were cultured to generate bmDC as described above and used in indicated experiments.

2. Bone marrow cells were isolated from FV-infected mice and cultured as described above. After 7-9 days of culture infected myeloid DC were isolated by CD11c⁺, CD11b⁺, and MAb 34 staining. Positive cells were separated by either flow cytometry or MACS technology.

**DC isolation from the spleen**

Splenic DC were obtained using a variation of the method described by Vremec et al. 14. Briefly, spleens from infected and uninfected mice were perfused with RPMI-FCS supplemented with 0.5 M ethylenediaminetetra-acid, 5mg/ml collagenase (Typ III, Worthington Biochmical ), DNase I (1mg/ml, AppliChem). Spleens were digested for 20min at 37°C. After centrifugation by 1200U/min for 10min the red cells were removed with lysis buffer. Then DC were further enriched using anti-CD11c microbeads (Miltenyi Biotech).

**Cell surface marker staining on FV-infected DC**

Expression of cell surface molecules on DC was quantified using following MAb: αCD11c-APC (HL-3), αCD54-FITC (3E2)αH-2D^d-FITC (34-2-12S), αl-A/I-E-FITC (2G9), αCD80-FITC (16-10A1), αCD86-FITC (GL1), αCD40-FITC (3/23) (BD PharMingen, Germany). For flow cytometry, 2x10⁵ DC were incubated with 1-5μg/ml MAb for 30min at room temperature,
washed twice in PBS/1%FCS and analyzed with a FACS Calibur (BD, Germany). Data were analyzed using Cell Quest Pro software.

For measuring antigen uptake and processing by FV-infected DC, DC were incubated for 2 hours with 0.5 µg/ml DQ Ovalbumin (Molecular Probes, Eugene, USA) and analyzed by flow cytometry and immunofluorescence microscopy.

**Cytokine measurement**

The cytokine production by bmDC was determined by measuring IL-12 in cell culture supernatants from CD11c-positive DC using a cytometric bead array (CBA Inflammation; BD PharMingen, Germany) and ELISA (eBioscience, San Diego, USA) according to the manufacturer's instructions.

**T-lymphocyte isolation and staining**

Naïve CD4⁺ T cells from spleens of DO11.10 mice were enriched to a purity of >95% as described by Gunzer et. al.¹⁵. Purified CD4⁺ T cells were cultured in RPMI supplemented with 5% FCS, 1x nonessential amino acids, 2mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate, 500nM 2-ME, 100U/ml penicillin/streptomycin.

CD25-positive CD4⁺ T cells were enriched to a >96 % purity by magnetic cell sorting.

**Analysis of cell-cell interactions within 3-D collagen gels**

DC-T-cell interactions within 3-D collagen gels were analyzed as described.⁶ Cell migration was monitored simultaneously by time-lapse microscopy using an Olympus BX61 microscope with an UAPO lens (20x340, NA 0.75) and a FView camera with AnalySIS software (SIS, Germany).
In some experiments 24h after starting the co-culture, 3-D collagen matrices were digested by type VII collagenase (30U/100 ml gel; Sigma Deisenhofen, Germany) for 30 min at 37°C and T cells were analyzed by flow cytometry. Expression of activation markers on T cells was quantified using following MAb against: αCD62-FITC (MEL-12), αCD69- PE (H1.2F3), αCD25-PE (PC-61), αCD44-FITC (IM7), αCD54-FITC (3E2) (BD PharMingen, Germany). In addition, regulatory T cells were quantified by FACS staining with Mab against: αCD4-PerCp (RM4-5), αCD25-PE (PC-61) (BD PharMingen, Germany) and αFoxp3-FITC (NatuTec, Frankfurt, Germany)

**Antigen-specific T cell proliferation**

BmDC from uninfected and FV-infected BALB/c mice were left untreated or were loaded with 0,1 µg/ml OVA-Ag (peptide 323-339) and titrated in triplicates one to three fold within 3-D collagen matrices. Afterwards 5x10⁴ antigen specific T cells (DO11.10) were added to obtain DC-T cells ratios of 1:10, 1:30, 1:90, and 1:270. Three days later, proliferating T cells were labeled by incorporation of [³H] thymidin (1µCi/Well) for 12h. The next day gels were digested by type VII collagenase (30U/100 ml gel; Sigma Deisenhofen, Germany) and thymidin incorporation was measured by liquid scintillation counting.

**T cell suppression assay**

For the proliferation assay, 2 x 10⁵ naïve T cells derived from Balb/c mice were labelled with CFSE, stimulated with anti-CD3 and anti-CD28, and analysed three days later by flow cytometry. For the suppression assay, 2 x 10⁵ CD4⁺ cells from T cell - DC cultures were simultaneously isolated and added to the proliferation assay. These CD4⁺ T cells were isolated
from three-day cultures (containing 250μl AIM-V 5%, FCS and IL-2 (100U)) of FACS-sorted FV-infected or uninfected DC that had been loaded with OVA peptides (0.1μg/ml), and MACS-enriched transgenic CD4+ T cells from DO11.10 mice (ratio: 1:1).

**Statistical analysis**

Differences between mean values were calculated by the student’s t-test for unpaired data or by one-way Annova test which was corrected by Dunnett’s multiple comparison correction if more than two groups were compared. P values <0.05 were considered as statistically significant.

**Results**

**Generation of FV-infected myeloid DC in vitro and detection of infected DC ex vivo.**

To study the functional properties of infected DC we generated DC from mouse bone marrow using GM-CSF and IL-4 12. However, unlike many other viruses 2, FV did not efficiently infect DC in vitro (data not shown). To solve this problem we took advantage of the ability of FV to infect bone marrow cells in vivo. It had previously been shown that about 20% of the bone marrow cells of C57BL/6 mice were infected at 2 weeks post FV inoculation 16. Thus, we infected BALB/c mice with FV and used their bone marrow cells to generate infected DC (Fig.1A). To visualize infected DC, electron microscopy analyses of DC cultures were performed. Figure 1B clearly shows the accumulation of virus particles in the cytoplasm of CD11c+ and CD11b+ (data not shown) myeloid DC after 9 days of in vitro culture. Up to 20% of the DC from BALB/c mice were infected with FV after 9 days of culture with GM-CSF and IL-4
Viral antigen on DC was detected using the monoclonal antibody 34 that recognizes the glycosylated form of Gag, expressed on the cell surface of both F-MuLV and SFFV infected cells. Synthesis of Gag protein, which is essential for virus particle formation, indicated that the DC were productively infected by FV. In order to show that infected DC can transmit virus in vitro and in vivo, sorted CD11c-positive DC from infected mice were adoptively transferred into naïve mice or plated on FV-susceptible Mus Dunni cells. Transfer of these DC induced severe erythroleukemia in susceptible BALB/c mice indicating that infected DC were able to established a productive FV infection in vivo (Fig.1C). In addition, FV-infected DC also transferred infectious virus to Mus Dunn i cells in vitro. In this assay up to 30% of the DC from cultures of FV-infected mice were found to be infectious (Fig.1D).

To determine whether the infection of DC in cell culture reflects the in vivo situation we stained spleen derived DC from acutely infected mice directly ex vivo for expression of FV glycosylated Gag. In susceptible BALB/c mice around 20% of the CD11c-positive DC were positive for viral antigen at 10 days post infection (Fig.1E). All FV-infected DC found in the spleen were of myeloid (CD11c+ CD11b+) or lymphoid (CD11c+ CD8+) origin but no infected plasmacytoid DC (CD11c low CD11b- B220+ or anti-mPDCA sorted cells) could be detected (data not shown). Thus, the infection rate of myeloid DC in vitro resembled the in vivo situation and therefore in vitro generated FV-infected myeloid DC were used to study the biological features of retrovirus-infected DC. Since there was no evidence of in vivo infection of plasmacytoid DC this cell subset was excluded from the subsequent phenotypic and functional analysis. Up to 15% of the lymphoid DC that were analyzed ex vivo from the spleen of infected mice were positive for FV (data not shown). However, since FV-infected lymphoid DC could not be generated in culture, the numbers of infected cells were two low to functionally characterize this DC subset.
FV infection interfered with maturation of DC but not with cytokine production

To analyze the consequences of FV infection of DC on the phenotype of these cells, the expression of cell surface markers was compared between infected and uninfected DC. We investigated co-stimulatory molecules, MHC molecules, and maturation markers expressed on murine CD11c+ DC in order to characterize the phenotypical consequences of FV infection. About 20% of the DC were infected in cell culture with the rest of the cells remaining uninfected (Fig.1A). Thus, both subsets were directly compared within the same DC culture from infected mice. After 9 days of culture with GM-CSF and IL-4 the CD11c-gated, uninfected DC generated from infected (Fig.2A) as well as uninfected mice (data not shown) were semi-mature and about 12% expressed the maturation marker CD83. In addition, 15 to 40% of the uninfected DC also expressed the co-stimulatory molecules CD40, CD80, or CD86 (Fig.2A). In contrast, only very few FV-infected DC from the same culture expressed co-stimulatory molecules or the maturation marker CD83 indicating that FV infection interfered with maturation of myeloid DC. Interestingly, no differences in the expression of MHC class I or II molecules were found between infected and uninfected DC (Fig.2A).

Immature DC can be matured and their antigen presentation capacity can be enhanced in vivo and in vitro by several different stimuli. Some of these stimuli, like type I interferons or murine retrovirus long terminal repeat, which has been shown to activate immune cells via toll-like receptors (TLR)-3, are present in vivo during an acute FV infection. We have investigated whether or not these stimuli can overcome the impairment in maturation of infected DC. Whereas IFNα and the TLR-3 ligand, poly(I:C), both enhanced CD86 expression on uninfected DC, no significant enhancement of the marginal CD86 expression was found after IFNα or
poly(I:C) stimulation of FV-infected DC, indicating that these stimuli could not overcome the FV-induced block in DC maturation (Fig. 2B). Comparable findings were made for the other two co-stimulatory molecules CD40 and CD80 and the maturation marker CD83 (data not shown).

Beside the phenotypic characterization of FV-infected DC we also studied their ability to produce cytokines. IL-12 and IL-10 are the most important cytokines produced by DC for activation and differentiation of T cells upon DC-T cell contact. After stimulation with poly(I:C), infected and uninfected DC produced similar amounts of IL-12 and IL-10 (Fig. 3) and no influence of the infection on cytokine production by DC was found. Stimulation of DC with IFNα resulted in only marginal production of cytokines independent of the infection status (data not shown). Interestingly, stimulation of DC with the TLR-9 agonist CpG1826 induced high concentrations of IL-12 and IL-10 in uninfected, but not in FV-infected DC (Fig. 3). However, the biological relevance of this finding remains unclear since CpG are present in bacterial DNA or in some viruses with a DNA genome but not in RNA viruses like the murine retroviruses.

**FV infection of DC changed the interaction of DC and naïve T cells**

In order to become activated by APC, T cells need to physically interact with APC and form a highly organized contact plane (immunological synapse). However, synapse formation is a dynamic process and is affected by the type and maturation state of APC. It is currently unclear whether the contact duration to APC determines the type of T cell activation, but at least some evidence suggests that prolonged APC-T cell contacts are required for the full activation of T cells.

To form an immunological synapse with T cells DC need to take up and process antigens. In order to show that FV-infected DC were not impaired in these functions they were incubated
with DQ ovalbumin, a self-quenched conjugate of OVA that exhibits bright green fluorescence upon proteolytic degradation. No difference in fluorescence labeling was found between infected and uninfected DC (data not shown) indicating that FV infection did not interfere with antigen uptake or processing. Next the ability of FV-infected DC to establish effective DC-T cell contacts was analyzed by measuring the contact duration between infected DC and naïve CD4+ T-cells. OVA-specific transgenic CD4+ T cells were isolated from DO11.10 mice and mixed in 3-D collagen gels with uninfected or FV-infected DC that had been loaded with the OVA antigen. Within the next 8h, cell movement and contact duration between T cells and DC were documented by video microscopy. Surprisingly, the contact duration between FV-infected DC and naïve CD4+ T cells was significantly prolonged (median contact duration of 20min) compared to uninfected DC (median contact duration of 12min) (Fig. 4A). This significant difference was the result of an increased number of very long contacts between infected DC and T cells. Significantly more contacts between FV-infected DC and T cells were longer than 38min (3 times the median contact duration between uninfected DC and T cells) (Fig. 4B) than contacts between uninfected DC and T cells. Examples of DC-T cell interactions with uninfected vs. infected DC are shown in Figures 4C and 4D. From these results we conclude that FV infection of DC can alter their interaction with CD4+ T cells.

**FV-infected DC preferentially expanded regulatory T cells**

Since FV infection prolonged the contact duration of DC with T cells it was important to determine whether this altered interaction resulted in sufficient T cell activation and proliferation. To this end, T cells were isolated from the 3-D collagen gels after 24h co-culture with DC and analyzed for their expression of activation markers. As negative controls, OVA-
specific T cells were cultured in the gel without DC or with DC that had not been loaded with the OVA peptide. In these control cultures, T cells were not activated as shown by high expression of CD62L but low expression of the activation markers CD69, CD25, CD54 and CD44 (Fig.5A). In contrast, if T cells were re-isolated from gels containing uninfected or infected DC loaded with the OVA peptide, CD62L expression was reduced and the expression of activation markers was strongly enhanced. The overall activation of the T cells was slightly lower after incubation with infected DC than with uninfected DC but the difference was not statistically significant. This result indicated that the OVA-loaded uninfected and FV-infected DC activated naïve T cells in an antigen-dependent manner.

Since naïve T cells were activated by FV-infected DC it was of interest whether or not infected DC could stimulate antigen-driven T cell proliferation. To test this we performed T cell proliferation assays. Uninfected DC loaded with the OVA peptide stimulated proliferation of OVA-specific CD4+ T cells (Fig.5B). At a DC-T cell ratio as low as 1:270 a significant increase in cpm was found when cultures containing uninfected DC loaded with OVA were compared with non-loaded DC (p<0.05). The magnitude of T cell proliferation was influenced by the number of DC added to the cultures. In contrast, proliferation was significantly reduced when FV-infected DC were used to stimulate the T cells. Only in the presence of numerous DC (DC:T cell ratios of 1:30 to 1:10) a weak T cell proliferation was detected with infected DC, whereas much fewer DC (ratios of 1:270 to 1:10) could induce proliferation when uninfected DC were used for antigen presentation.

Thus, although FV-infected DC were able to activate naïve T cells this activation did not lead to efficient proliferation of the cells in vitro. This phenomenon might contribute to the virus-induced immunosuppression observed in acute FV-infected mice. To test this hypothesis we
analyzed whether FV-infected DC induced or expanded regulatory CD4+ T cells, which have been shown to be associated with immunosuppression in FV infection 25. As a marker for Treg cells we used the transcription factor Foxp3 26. After 24h of co-culture with uninfected or FV-infected DC, T cells were isolated from the 3-D collagen gels and analyzed for Foxp3 expression. Less than 14% of the T cells that were mixed with OVA-loaded uninfected DC expressed Foxp3, which was indistinguishable from CD4+ T cells prior to co-culture (Fig.6A). However, a significant increase of Foxp3+ T cells was found when antigen-loaded, FV-infected DC were used for antigen presentation to T cells (more than 26% of the total CD4+ T cells were positive) (Fig.6A). Almost all of the Foxp3+ T cells that were expanded by the infected DC were also positive for the Treg cell markers CD25 and GITR (over 97%), and about 30% were positive for CD103 (data not shown). The expansion of T cells with a phenotype of Treg cells correlated with the ability of such cells to suppress the proliferation of effector T cells. Total CD4+ T cells that were activated by FV-infected DC and contained about 26% Foxp3+ cells partly inhibited the proliferation of naïve CD4+ T cells that were stimulated by antibodies against CD3 and CD28 (Fig.6B). Thus, a sufficient number of Treg cells was present in these cultures that could suppress other T cell responses. In contrast, the CD4+ T cells from cultures with uninfected DC showed no suppressive activity. Antigen presentation by infected DC to the naïve T cells was required for the expansion of the Treg cells, as no suppressive T cells were found without loading the DC with the OVA peptide.

Thus, FV-infected DC were able to expand the population of Treg cells during contact to naïve T cells and this activity was dependent on the presentation of the congnate antigen to specific T cells.
Treg cells that were expanded by infected DC originated from naïve CD25+ natural Treg cells

To analyze whether or not the expanded Treg cells were originated from CD25+ T cells we depleted these cells prior to the cultivation of FV-infected DC with naïve T cells. Stimulation of the OVA-specific CD4+CD25- T cells with OVA-loaded DC resulted in very little expression of Foxp3 in the T cells (Fig.7A) and no difference was found between cultures with infected versus uninfected DC. In contrast, after culturing total CD4+ T cells and infected DC a population of Foxp3+ cells was detectable. As also shown in Figure 6 this population was expanded after the culture with FV-infected DC in comparison to uninfected DC. These results correlated with the results from a proliferation assay with CD25-depleted CD4+ T cells (Fig.7B). As shown before, FV-infected DC loaded with OVA-peptide induced only a very weak proliferation of OVA-specific CD4+ T cells (Fig.7B). However, if the CD25+ T cells were removed from the CD4+ T cells the infected DC induced an OVA-specific T cell proliferation that was similar to that induced by uninfected DC. Thus, naïve CD25+CD4+ T cells, a cell population that has been described as natural Treg cells 27, were required for the expansion of Foxp3+ immunosuppressive Treg cells by FV-infected DC.

To test the hypothesis that the expansion of Treg cells after interaction with infected DC was associated with the significant increase in long contacts between naïve T cell and infected DC, we did the following control experiment in which the contact duration of natural Treg cells and DC was determined. Enriched CD4+CD25+ natural Treg cells, which were negative for other activation markers, like CD69 and CD44, but positive for Foxp3 (data not shown), were isolated from naïve OVA-specific T cells and incubated with OVA-loaded uninfected DC. In these experiments we found significantly prolonged contact durations between these cells compared to
contacts between DC and CD4⁺CD25⁻ T cells (Fig. 4E). Interestingly, the number of very long contacts was significantly increased for the natural Treg cells (Fig.4F). This was not associated with their CD25 expression because it has been previously shown that pre-activated helper T cells do not form longer contacts to DC than naïve CD4⁺ T cells 28. The results suggest that DC can establish long and stable contacts to Treg cells, which might be critical for their expansion and functional activation.

Discussion

In the current study we show that Friend retrovirus productively infects myeloid DC and changes their phenotype and interaction with T cells. Several different viruses are able to infect DC in their host, which leads in most cases to an impairment in the induction of immune responses by DC 2. Thus, it has been discussed that viral infections that induce severe immunosuppression in the host, like HIV or measles virus, might be associated with an infection of DC in general 29. Infection of DC often results in impaired maturation and cytokine production by the DC, which directly interferes with proper antigen presentation to and activation of T cells. FV does also induce severe immunosuppression during infection 13 and we demonstrate here that FV infects DC. The infection resulted in a large reduction in the expression of co-stimulatory molecules, a characteristic of immature DC. It has been shown that immature DC are impaired in the formation of immunological synapses and fail to properly activate T cells 23. In contrast, we found that the mean contact times between FV-infected DC and naïve T cells were prolonged in comparison to those of uninfected DC and CD4⁺ T cells and that infected DC were able to induce activation of T cells. It has to be pointed out that not every single contact between FV-
infected DC and T cells was prolonged but only the number of T cells that had very long contacts was significantly increased. It remains to be determined which molecules were involved in stabilizing the long contacts of infected DC. Since the classical co-stimulatory were obviously not involved other molecules that were reported to stabilize the DC – T cell synapse, like LFA-1 or semaphorins, have to be investigated in the future.

Using time-lapse video microscopy of T cell – DC interactions in collagen gels as an experimental model, one routinely observes two distinct modes of interaction: the majority of T cell – DC interactions are short-lived and transient, whereas another part of these contacts is of prolonged duration (>38min), with only very few interactions of immediate duration. It has been shown for CD8+ T cells that long-lasting T cell – DC interactions induce T cell activation whereas short contacts induce tolerance, but clear experimental evidence for this scenario in CD4+ T cell – DC interaction is still lacking. In our own experiments, prolonged T cell – DC contacts induced by pharmacological activation of beta2 integrins on DC resulted in impaired rather than augmented T cell activation, suggesting that T cell activation and APC-T cell contact duration are not strictly proportional.

Interestingly, FV-infected DC activated CD4+ T cells but were not able to induce significant antigen-specific T cell proliferation. This suggests that at least some of the T cells that were stimulated by the FV-infected DC cells were not typical helper T cells that usually proliferate upon stimulation with their cognate antigen. In fact, it has previously been shown that Treg cells do not proliferate in vitro in the absence of exogenous IL-2 and that they efficiently suppress the proliferation of helper T cells within the same culture. We demonstrate here that FV-infected DC induce or expand CD4+ T cells that express the Treg cell-associated marker Foxp3, in an antigen-dependent manner. Thus, FV infection might influence the mode of T cell stimulation by
DC. It has previously been shown that immature DC preferentially induce Treg cells that are able to suppress other T cell responses\textsuperscript{34-38}. Interestingly, CD80/CD86 expression by the immature DC was not required for the induction of the Treg cells\textsuperscript{36}, supporting results from our experiments. At the moment we can only speculate whether the prolonged contacts between FV-infected DC and T cells are directly associated with the induction or expansion of Treg cells. It is conspicuous that the percentages of T cells with significantly longer contacts to DC as well as the percentages of Foxp3+ T cells were both about 15% higher when we compared cultures with FV-infected to those with naïve DC, suggesting that Treg cells might establish longer contacts to DC than other T cells. This hypothesis was supported by the control experiment using CD4+CD25+ natural Treg cells. Here we found significantly prolonged contact durations between these cells compared to contacts between DC and CD4+CD25- T cells (Fig. 4E). Again, especially the number of very long contacts was significantly increased for the natural Treg cells. Overall our results suggest that DC can establish long and stable contacts to Treg cells. This is in line with findings from a diabetes model in which persistent Treg cell – DC contacts were demonstrated \textit{in vivo}\textsuperscript{39}, and with a recent report showing that long contacts of naïve CD4+ T cells to antigen presenting B cells resulted in Treg cell induction\textsuperscript{40}.

Taken together, the infection of DC by FV plays a substantial role in DC - T cell interactions, the stimulation of different T cell populations and the induction of immunosuppression. Virus-infected, immature DC might be responsible for the induction or expansion of Treg cells which are found during acute infection of mice with FV\textsuperscript{41} or monkeys with simian immunodeficiency virus\textsuperscript{42}. Interestingly, at least in our \textit{in vitro} experiments the Treg cells originated from the pool of natural CD25+ Treg cells. The expanded Treg cells then suppressed CD8+ T cell responses early during infection before the virus could be completely eliminated and consequently
contribute to chronic infection \(^{25}\). In fact, treatment of FV-infected mice with immunostimulatory CpG oligodeoxynucleotides (CpG-ODN) can partly reverse the general immunosuppression observed during acute FV infection \(^{43}\). In addition, we have been able to demonstrate recently that CpG-ODN induce at least partial maturation of FV-infected DC \textit{in vitro} and increase the capacity of infected DC to activate naïve CD8+ T cells \(^{44}\). These results may indicate innovative strategies for the treatment of immunosuppressive viral infections.

Along these lines our current findings in the Friend virus model might have relevance for human viral infections as well. For example, HIV also infects DC and can interfere with their function and maturation \(^{45-48}\). Furthermore, similar to FV-infected DC, HIV-infected DC are inactive in stimulating T cell proliferation \(^{49}\). It has recently been reported that semi-mature DC, which stimulate T cell tolerance rather than immunity, accumulate in the lymph nodes of HIV-infected patients \(^{50}\). However, in this study it remains unclear whether or not these semi-mature DC were infected by HIV. Several reports have clearly demonstrated that HIV-infected patients have expanded populations of Treg cells, which can suppress HIV-specific T cell responses \textit{in vitro} \(^{51-54}\) and correlate with the inability of the cellular immune response to control viral replication \(^{53,55}\). Taken together, viral infection of DC might also lead to the induction or expansion of Treg cells in HIV-infected humans and subsequently results in acute immunosuppression, loss of immune control of viral replication, and the development of chronic infection. Therapeutic means to mature virus-infected DC cells might be a new general strategy to successfully treat chronic retroviral infections.
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Figure legends

Figure 1: Friend Virus infection of myeloid DC in vitro and in vivo

Myeloid DC were generated from the bone marrow of FV-infected BALB/c mice and analyzed for viral infection. A) CD11c-gated DC were stained for FV glycosylated Gag-Protein using monoclonal antibody 34 (Ab 34). A flow cytometric analysis representative for more than 20 independent DC cultures is shown (mean: 21.2% ± 0.8%). The mean percentage of positive cells (FV-infected cells) is given in the upper right quadrant. B) Electron microscopy picture of virus particles in an infected DC. Virus particles are visible as numerous small electron dense bodies at the margin of the cytoplasm (arrow). Transmission electron microscopy, original
magnification x 28,000. C) FV-induced splenomegaly in BALB/c mice at 2 weeks post transfer of 3.5x10^5 infected DC (right). D) Quantification of infected DC using Mus dunni cells. 1x10^5 DC were co-cultivated with the indicator cells which were subsequently stained for infectious centers (IC).

Representative results for more than 5 independent experiments are shown. The uninfected DC were generated from naïve mice.

E) Myeloid DC were isolated from the spleen of FV-infected BALB/c mice and analyzed for viral infection directly ex vivo. CD11c-gated DC were stained for FV glycosylated Gag-Protein using monoclonal antibody 34 (Ab 34). A flow cytometric analysis representative for 5 individual mice is shown (mean: 22.3% ± 1.5%). The mean percentage of positive cells (FV-infected cells) is given in the upper right quadrant.

**Figure 2: Expression of co-stimulatory and MHC molecules on FV-infected DC.**

A) Myeloid DC were generated from the bone marrow of FV-infected BALB/c mice and analyzed for the expression of cell surface molecules. CD11c-gated DC were stained for FV glycosylated Gag-Protein using monoclonal antibody 34 (Ab34) and for co-stimulatory molecules, maturation maker CD83, or MHC molecules. The infected cells are in the right quadrants whereas uninfected cells are shown in the left quadrants. The different populations were from the same culture of DC generated from an infected mouse. The percentage of cells positive for the different DC markers in both the infected and uninfected populations of DC is
given in the upper quadrants. A flow cytometric analysis representative for more than 10 independent DC cultures is shown.

B) Myeloid DC were generated from the bone marrow of FV-infected or uninfected BALB/c mice and stimulated in vitro with IFNα (1000U/ml) or Poly(I:C) (100µg/ml). Afterwards, CD11c+ gated DC were stained for the expression of the co-stimulatory molecule CD86 as a surrogate marker for DC maturation. The histogram on the left shows the mean fluorescence intensity (MFI) of uninfected cultures whereas the right histogram shows the results for FV-infected DC cultures. The gray curves represent the isotype control for the CD86 staining. The lower histogram shows the CD86 staining of uninfected or infected DC that were not treated (no IFN or Poly(I:C)). A flow cytometric analysis representative for 6 independent DC cultures is shown.

Figure 3: Production of IL-12 and IL-10 by FV-infected DC.

Myeloid DC were generated from the bone marrow of FV-infected (inf DC) or uninfected (DC) BALB/c mice and stimulated in vitro with CpG 1826 oligodeoxynucleotides (6µg/ml) or Poly(I:C) (100µg/ml). Infected DC were enriched by FACS-based cell sorting. As negative controls DC were stimulated with an oligodeoxynucleotide without CpG motif (ODN-control) or left non-stimulated. After 24 hours supernatans from stimulated cultures were analyzed by ELISA for the concentration of IL-12 (A) or IL-10 (B). Cumulative data from 3 independent experiments are shown.
Figure 4: Contact duration between FV-infected DC and naïve T cells

DC-T-cell interactions were analyzed within a 3-D collagen gel. Transgenic CD4+ T cells from DO11.10 mice were isolated and mixed with OVA-antigen loaded DC in a 10:1 ratio. A) Contact duration times (min) between uninfected DC (filled circles) or FV-infected DC (filled squares) and CD4+ T cells are shown. The median for both groups (gray line) was statistically significantly different (p<0.0001). Shown are the cumulative results from 4 independent experiments with similar outcomes. The total number of measured contacts were: uninfected DC = 330 and FV-infected DC = 329. B) In addition, cell contacts between uninfected (white) and FV-infected (black) DC and CD4+ T cells that lasted longer than 38 min (3 times the median of the contact times between T cells and uninfected DC) were counted. Cumulative results from 4 independent experiments are shown. The difference between both groups was statistically significant (p=0.0005). C) Representative example of short cell contacts between one uninfected DC and two naïve CD4+ T cells. The time point when the picture was taken after initiation of the culture is given. D) Representative example of a long contact between one FV-infected DC and two naïve CD4+ T cells.

(E) Contact duration between DC and natural regulatory T cells. DC-T-cell interactions were analyzed within a 3-D collagen gel. Naïve CD25+ (Foxp3+ but CD69- and CD44-) or CD25- transgenic CD4+ T cells from DO11.10 mice were isolated and mixed with OVA-antigen loaded uninfected DC in a 10:1 ratio. Contact duration times (min) between DC and CD4+CD25- (left) or CD4+CD25+ (right) are shown. The median for both groups (gray line) was statistically significantly different (p<0.0001). Shown are the cumulative results from 3 independent experiments with similar outcomes. The numbers of measured contacts were: CD4+ = 167 and CD4+CD25+ = 106. (F) In addition, cell contacts between CD4+CD25- (white) and CD4+CD25+
(black) T cells and DC that lasted longer than 3 times the median of the contact times between the total CD4+ T cells and uninfected DC were counted. Cumulative results from 3 independent experiments are shown.

Figure 5: Activation and proliferation of T cells after co-cultivation with FV-infected DC

A) Naïve TCR tg CD4+ T cells from DO11.10 mice were incubated with infected or uninfected DC in a 3-D collagen gel. 24h later gels were digested and T cells were analyzed for activation marker CD62L, CD69, CD25, CD54 or CD44 surface expression by flow cytometry. T cells were mixed with uninfected (white bar) or FV-infected DC (black bar), which were loaded (+Ova) or not (-Ova) with OVA peptide. T cells without DC severed as negative control (grey bar). The mean fluorescence intensity or the percentage of positive stained cells is shown. Cumulative data from 5 independent experiments are being presented.

B) FV-infected (filled square) and uninfected (filled circle) DC were loaded with 0.1µg / ml OVA peptide and titrated onto naïve TCR tg CD4+ T cells from DO11.10 mice in a 3-D collagen gel. Cells were incubated for three days and then pulsed with [3H] thymidin overnight to determine T cell proliferation. All assays were performed in triplicate. Cultures with DC that were not loaded with peptide served as negative controls (open symbols). Stars indicate statistically significant differences (p<0.0001) between cultures with FV-infected versus uninfected DC loaded with OVA antigen. Representative results from four independent experiments are shown.
Figure 6: Induction of Treg cells after cocultivation with FV-infected DC

(A) Naïve TCR tg CD4+ T cells from DO11.10 mice were incubated with DC in a 3-D collagen gel. 24h later gels were digested and T cells were analyzed for the intracellular expression of the Treg cell marker Foxp3 by flow cytometry. T cells were mixed with uninfected (white bar) or FV-infected DC (black bar), which were loaded with OVA (+Ova) or not loaded with the OVA peptide (-Ova). T cell prior to co-culture served as negative control (grey bar). The percentage of positively stained cells in the total CD4+ T cells is shown. Cumulative data from 7 independent experiments are shown. Differences between cultures with FV-infected versus uninfected DC were statistically significant (p=0.0061).

(B) Naïve TCR tg CD4+ T cells from DO11.10 mice were incubated with FV-infected or uninfected DC for 3 days. After culture T cells were isolated and added to a polyconal CD4+ T cell proliferation assay.

Left: As negative control 2 x 10^5 non-stimulated CD4+ T cells were labeled with CFSE and analyzed by flow cytometry three days later.

Middle: 2 x 10^5 naïve CD4+ T cells were stimulated with antibodies against CD3 and CD28 and labeled with CFSE three days prior to analysis. At the same time 2 x 10^5 total CD4+ T cells that were stimulated previously by uninfected DC were added. However, adding non-stimulated CD4+ T cells to the culture had no influence on the proliferation of the anti-CD3, anti-CD28 stimulated T cells (data not shown). The mean fluorescence intensity of three independent tests was 1155 ± 21.6.

Right: 2 x 10^5 naïve CD4+ T cells were stimulated with antibodies against CD3 and CD28 and labeled with CFSE three days prior to analysis. At the same time total 2 x 10^5 CD4+ T cells
containing about $5 \times 10^4$ Foxp3$^+$ cells that had been stimulated previously by FV-infected DC were added. The mean fluorescence intensity of three independent tests was $1842 \pm 51.7$.

M1 shows the percentage of non-proliferating cells in each culture. Representative data for 3 independent experiments with similar results are shown. The difference in the mean fluorescence intensity between the groups labeled “uninfected DC” and “infected DC” were statistically significant (p<0.0001).

**Figure 7: Expansion of Treg cells by FV-infected DC depends on naïve CD4$^+$CD25$^+$ T cells**

A) Naïve TCR tg CD4$^+$ (purity: >97%) or CD4$^+$CD25$^-$ T cells (purity: >96%) from DO11.10 mice were incubated with uninfected (thin small grey line) or FV-infected DC (fat black line) in a 3-D collagen gel. 72h later gels were digested and T cells were analyzed for the intracellular expression of Foxp3 by flow cytometry. The experiments were repeated three times with comparable results and the results from representative cultures are shown.

B) $5 \times 10^3$ uninfected (white) or FV-infected (black) DC were loaded with the OVA peptide and co-cultured with $5 \times 10^4$ naïve TCR transgenic total CD4$^+$ or CD4$^+$CD25$^-$ T cells from DO11.10 mice in a 3-D collagen gel. Cells were incubated for three days and then pulsed with [$^3$H] thymidin overnight to determine T cell proliferation. Assays were done in triplicates and mean counts per minute (cpm) and standard deviations are shown. Differences between cultures with CD4$^+$ versus CD4$^+$CD25$^-$ were statistically significant (p<0.0001).
References

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Fig. 3

A

B

pg/ml IL-12

Poly(I:C)  inf DC  DC  inf DC  DC  DC

CpG 1826

ODN control

pg/ml IL-10

Poly(I:C)  inf DC  DC  inf DC  DC  DC

CpG 1826

ODN control
Fig. 4

A

B

E

C

D

F

Median contact duration (min)

% of long contacts

Median contact duration (min)

uninfected infected

uninfected infected

CD4+ CD4+/CD25+

uninfected DC

infected DC

0 min 8 min 18 min 23 min

0 min 20 min 48 min 63 min

p<0.0001

p<0.0001

p<0.0005

p=0.0005

p=0.00295

p<0.0001

CD4+ CD4+/CD25+
Fig. 7

A

CD4\(^+\)  

\[\text{counts}\]

CD4\(^+\)/CD25\(^-\)

\[\text{Foxp3}\]

\[\text{infected}\]  

\[\text{uninfected}\]

B

\[p<0.0001\]

\[\text{uninfected}\]  

\[\text{infected}\]

\(\text{CD4}^+\)  

\(\text{CD4}^+\)  

\(\text{CD4}^+\)/\(\text{CD25}^-\)
Friend retrovirus infection of myeloid dendritic cells impairs maturation, prolongs contact to naive T cells, and favors expansion of regulatory T cells

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