Regulation and subversion of HPV16-specific immunity in cancer patients

Piersma, S.J.

Chapter 6

Sytse J. Piersma
Jeanette M. van der Hulst
Kitty M.C. Kwappenberg
Renske Goedemans
Caroline E. van der Minne
Sjoerd H. van der Burg

Healthy donor derived IL-10 producing influenza virus M1-specific CD4 T cells encompass FOXP3+ and FOXP3- T-regulatory cells as a consequence of previous acute infection

submitted

Abstract. Control and termination of an infection with Influenza A virus is associated with IL-10 production in mouse models. IL-10 plays an essential role in dampening virus-specific immune responses in order to limit collateral damage. Virus-specific CD4+ T cells are an important source of IL-10. This study investigated whether the population of IL-10 producing influenza-specific CD4+ T cells comprised of regulatory T cells (Treg) as they are potent suppressors of the adaptive immune response. Influenza-specific IL-10 producing T cells were detected in all human donors displaying influenza-specific immunity. Isolation of Matrix 1 protein-specific IL-10 producing T-cell clones revealed that a substantial proportion of the IL-10 producing T-cell clones displayed the capacity to suppress the proliferation of effector cells, functionally identifying them as Tregs. Both FOXP3+ and FOXP3- CD4+ Tregs were isolated and all were able to exert their suppressive capacity when stimulated with their cognate antigen, including influenza virus-infected APC. The isolated Tregs suppressed the IL-2 production of influenza-specific T-helper cells as well as prevented the upregulation of the high-affinity IL-2 receptor on CD8 effector cells. As these influenza-specific Tregs can dampen the immune response by interfering at both sides of the IL-2 regulatory pathway they may be crucial in preventing immunopathology, which otherwise results from an exuberant immune response after acute influenza infection.

Introduction

Influenza A virus infections have caused many pandemics (1). Infections with this virus are acute and characterized by acute onset of fever, myalgias and respiratory symptoms (2). Data in experimental mouse models showed that immune control of influenza infection is associated with the production of IFNγ at the start and then followed by a peak in IL-10 when viral infection becomes controlled (3).

IL-10 is well known for its anti-inflammatory effects and is known to limit and ultimately terminate inflammatory responses (4). The prominent role of IL-10 in limiting collateral damage is well illustrated in IL-10 deficient mice, as they spontaneously develop chronic enterocolitis when not housed under specific pathogen free conditions (5). These mice also develop lethal liver pathology as a result of uncontrolled inflammation when challenged with a non-virulent strain of the parasite Toxoplasma Gondii (6). IL-10 can be produced by several members of both the innate and the adaptive immune system (7). In a Cre/loxP mouse model it was shown that the prevention of immunopathology after challenge with Toxoplasma Gondii was dependent on the IL-10 production by CD4+ T cells (8). Moreover, a recent study shows that IL-10 produced by FOXP3+ regulatory T cells (Treg) is essential for limiting inflammation at environmental surfaces (9). FOXP3+ Treg cells can be generated both in the thymus and in the periphery (10). Generation of Tregs in the periphery has been well demonstrated in mouse models (11-13). Moreover, pathogen-specific FOXP3+ Tregs in humans have been isolated in the context of chronic infections and viral induced cancer (14-17). Evidence is starting to accumulate that Tregs also may have an important role in preventing immunopathology which otherwise would have been caused as result of immune control of infections (18, 19). However, the specificity of such Tregs has not been addressed.

In mice, influenza-specific immunity not only comprises influenza-specific CD4+ Th1 cells, but also a subset of influenza-specific CD4+ T cells able to produce IFNγ and IL-10, simultaneously (20). Interestingly, this cytokine profile resembles that of previously described adaptive Tregs (14, 16). Moreover, analysis of the influenza-specific CD4+ T-cell response by MHC-class II tetramers revealed a small but discernible population of CD4+FOXP3+ T cells, suggesting but not showing the induction of influenza-specific Tregs as result of influenza infection (16).

Based on these data, we set out to study the influenza-specific CD4+ T-cell response in healthy individuals. T-cell immunity to influenza is characterized by both the production of IFNγ and IL-10. The majority of IL-10 producing T cells were also capable of producing IFNγ but displayed an immunosuppressive signature, as isolated T-cell clones were able to suppress CD4+ and CD8+ T cells when stimulated with influenza. These data show that IL10-producing Tregs are induced during acute infections and it is highly likely that they play a role in preventing immunopathology.

Materials and Methods

Subjects and antigens. Anonymous healthy blood bank donors participated in this study after written informed consent. Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-amidotrizoate density gradient centrifugation. Peptides spanning the whole M1 protein consisted of 16 peptides with a length of 30 amino acids, and an overlap of 15 amino acids (C-terminal peptide with an overlap of 18 aa), the sequence was derived from influenza A/PR/8/34. Recombinant M1 and HPV16 E6 protein (the latter served as control protein) were produced in E. coli as described previously (21). Live influenza A/Wisconsin/67/2005 was kindly provided by W.M. Liu (NVI, Bilthoven, the Netherlands).

Flow cytometry. Fluorescent-labeled antibodies used were CD4 PE (Clone SK3), CD4 APC (Clone RPA-T4), CD8 PERCP-CY5.5 (Clone SK1), CD25 APC (Clone M-A251), CD69 FITC (Clone L78), CD137 APC (Clone 4B4-1) and IL-2 PE (Clone MQ1-17H12) and were obtained from Becton Dickinson (USA). FOXP3 was stained using the FOXP3 PE staining kit (clone PCH101) according to manufacturer protocol (eBiosciences, USA). The previously described clones C148.31 and C271.9 were used as reference to determine the cut-off level (10, 14). Samples were analyzed by fluorescent activated cell sorting (FACS) using FACS Calibur (Becton Dickinson) and data was analyzed using Cell Quest pro (Becton Dickinson) and FlowJo software (Tree Star).

Generation and culture of M1-specific T-cell lines and isolation of M1-specific T-cell clones. To generate M1-specific T-cell lines PBMC were cultured in IMDM (BioWhittaker, Belgium) supplemented with 10% human AB serum (PAA laboratories, Austria) and 10% T-Cell Growth Factor (TCGF, Zeptometrix, USA) and were stimulated with 5 μg/ml peptide pools containing the first 8 or the last 8 overlapping peptides. After 2 weeks of culture the reactivity against M1 peptides and recombinant protein was assessed. Positive cultures were stimulated for 4 hours with peptide-loaded autologous monocytes and were subsequently enriched for IL-10 producing cells according to manufacturer protocol (IL-10 secretion assay; Miltenyi Biotech, Germany). Directly after enrichment T-cell clones were isolated as described before (22). After limiting dilution, T-cell clones were tested for their specificity and maintained in IMDM supplemented with 10% FCS and 10% TCGF. Clonality of the isolated T-cell clones was assessed using a Beta Mark TCR Vβ Repertoire kit according to manufacturer protocol (Beckman Coulter, USA) and analyzed by FACS. T-cell clones were expanded every 2-3 weeks using a mix of IMDM supplemented with 10% FCS and 10% TCGF, irradiated PBMC from 5 different donors and irradiated autologous B-LCL loaded with 5 μg/ml cognate peptide.

T-cell stimulation assays. T-cell cultures (25,000-50,000 cells/well) were tested on pulsed autologous monocytes or irradiated autologous B-LCL for the recognition of M1 peptides (5 mg/ml) and protein (10 mg/ml) in triplicate in a 3-day proliferation assay (22). For generation of monocytes, PBMC were seeded in flat bottom 96-wells plates (Greiner bio-one, the Netherlands) and adherent PBMC were cultured for 3 days in X-vivo medium (BioWhittaker) containing 800 IU/ml GM-CSF (Invitrogen, UK) before use. For experiments with influenza virus, autologous monocytes were infected with 5 x 10^5 PFU/ml A/Wisconsin/67/2005 for 5 hours before addition of M1-specific T-cell clone. After 48 hours supernatant was harvested and stored at -20°C for cytokine analysis. During the last 16 hours of culture 0.5 mCi/well [^{3}H]thymidine (Perkin Elmer, USA) was added to measure proliferation (21). Antigen-specific IFNγ and IL-10 production was measured by ELISA as according to manufacturer protocol (Sanquin, the Netherlands). For the analysis of cytokine production on a single-cell level T-cell clones were stimulated for 4 hours with peptide-loaded autologous monocytes and were subsequently stained for IL-10 and IFNγ according to manufacturer protocol (IL-10 and IFNγ secretion assay; Miltenyi Biotech) and analyzed by FACS.

T-cell suppression assays. For anti-CD3 based suppression assays responder CD4+CD25- cells were isolated from PBMC ad described before (14). CD8+ lymphocytes were isolated using magnetic Dynal beads (Invitrogen, USA) and used as CD8+ responder cells where indicated. 1 x 10^5 responder cells were cultured with M1-specific T-cell clone at different ratios in the presence of 1 x 10^4 irradiated B-LCL and 1 mg/ml agonistic anti-CD3 antibody (OKT-3, Ortho Biotech, USA). Proliferation and cytokine production was determined as described above. Cell surface activation markers were stained 24 hours after stimulation and analyzed by FACS.

For antigen dependent suppression experiments CD4+CD25- responder cells were stained with 5 mM carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) for 15 minutes at 37°C. M1-specific T-cell clone was stained with PKH26 according to manufacturers' protocol (Sigma), treated with Mitomycin C (50 mg/ml; Kyowa, Japan) for 1 hour and irradiated (2000 Rad) to prevent proliferation of the clone. 1 x 10^5 of both fluorescent labeled T-cell populations were cocultured in IMDM containing 10% FCS and IL-2 (300 IU/ml) in the presence of a mix of 1 x 10^4 irradiated B-LCL from 5 different donors, which included B-LCL autologous to the M1-specific T-cell clone as antigen presenting cell for the clone. In experiments using influenza virus, autologous B-LCL were infected overnight, hereafter the B-LCL were irradiated and washed extensively. After 4-6 days of culture, the allo-specific proliferation of responder T cells was analyzed by FACS.

For measurement of suppression on IL-2 production CFSE-labeled D1.50 was cocultured with the indicated M1 specific T-cell clone at a 1:1 ratio together with autologous B-LCL in the presence of 50 IU/ml IL-2. The Treg clone was prestimulated with 5 μg/ml cognate peptide. After 24 hours D1.50 was stimulated with 5 μg/ml cognate peptide, 1 h later 10 μg/ml Brefeldin A (Sigma-Aldrich) was added. After overnight incubation, the cells were fixed, permeabilized, and stained for CD4 and intracellular IL-2 as described earlier (23). The percentage of IL-2-producing cells was analyzed by FACS.

Results

Influenza M1-specific T-cell responses are accompanied both by IFNγ and IL-10. The immune response to influenza infection in mice is characterized by a first wave of IFNγ and is followed by IL-10 when the viral infection is controlled (3). This immune response not necessarily reflects the contraction of populations of T cells (eg. Th1 and Th2) as one single influenza-specific CD4 T cell can produce both IFNγ and IL-10 in mice (20). To study whether similar responses could also be observed in humans, the influenza specific T-cell response in healthy individuals was analyzed. Freshly isolated PBMC from healthy blood bank donors were stimulated with a pool of influenza matrix 1 (M1) peptides in order to expand the influenza M1-specific response as we had previously observed that M1-specific T cells can be detected in the majority of individuals, directly ex vivo (21, 24-26). M1-specific responses were detected against multiple peptides, indicating that a broad T-cell response was mounted against influenza in these donors (Figure 1A). T-cell reactivity to some of the M1-specific peptides comprised the production of both IFNγ and IL-10. In total we

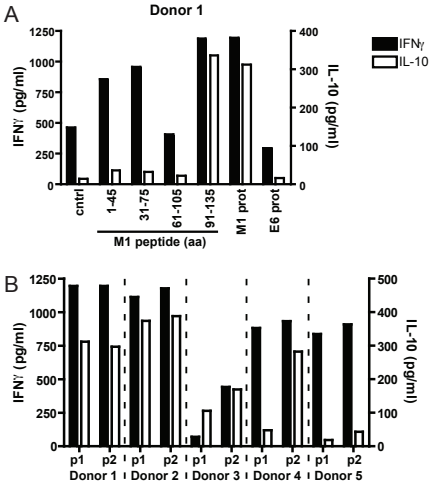

Figure 1. Influenza-specific T-cell responses in healthy donors are accompanied by IFNγ and IL-10 production. PBMC were stimulated with overlapping influenza M1 peptides (5 μg/ml) in 2 pools (p1=aa 1-135; p2=aa 121-252) and after 2 weeks the reactivity against M1 peptides and recombinant protein was assessed. The IFNγ (filled bars) and IL-10 (open bars) secreted by the T-cell cultures was measured in the culture supernatant by ELISA. A) Reactivity against multiple epitopes within the bulk culture of Donor 1. M1 prot = influenza M1 protein, E6 prot = HPV16 E6 protein. B) IFNγ and IL-10 production of T-cell bulk cultures from 5 different donors stained with recombinant influenza M1 protein pulsed APC.

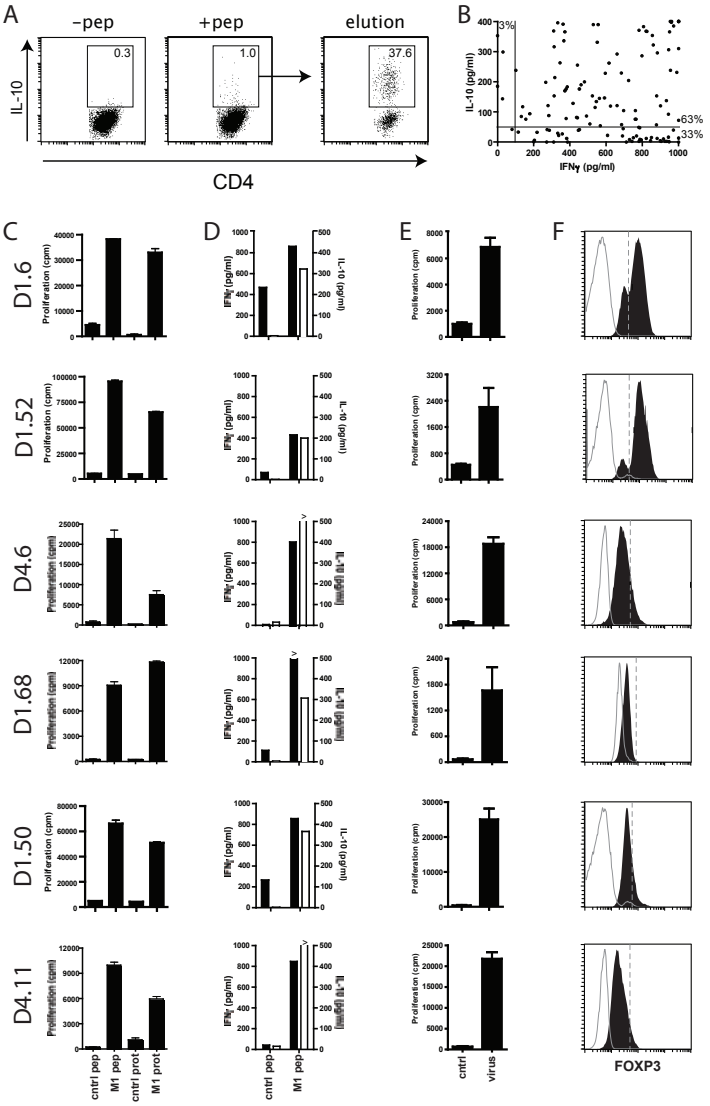

Figure 2. Isolation, specificity and phenotype of IL-10 producing M1-specific T-cell clones. A) Example of enrichment of IL-10 producing cells using cytokine capture assay. The number indicates the percentage of IL-10 producing CD4+ T cells. B) M1-specific IL-10 versus IFNγ production of individual M1-specific T-cell clones. Each dot indicates one T-cell clone. C) Individual M1-specific T-cell clones react against both M1 peptide and recombinant protein. D1.6 recognized M1 pep 31-60, D1.52 recognized M1 peptide 1-30, D4.6 recognized M1 peptide 46-75, D1.68, D1.50 and D4.11 recognized M1 peptide 91-120. D) Proliferation-associated IL-10 and IFNγ production of individual clones measured by ELISA. E) Reactivity of M1-specific T-cell clones against influenza virus-infected or non-infected (cntrl) monocytes. F) FOXP3 expression by T-cell clones measured 21 days after their last specific stimulation. Black areas represent FOXP3-specific staining, grey lines represent isotype controls. Established T-cell clones C148.31 and C271.9 were used as reference to determine cut-off level (10, 14). The cut-off level is indicated by the vertical dotted lines.

analyzed 10 donors, of which 5 showed M1-specific responses. In all cases the responding T cells reacted both against peptide and recombinant protein pulsed APC, showing that the M1-specific T cells recognize naturally processed epitopes. Moreover, the responses were accompanied by both IFNγ and IL-10 in all cases (Figure 1B).

The M1-specific IL-10 producing CD4+ T-cell population comprises of both FOXP3+ and FOXP3- cells. To characterize the influenza-specific IL-10 producing T cells at the single cell level, the IL-10 producing influenza-specific T-cell population was enriched by magnetic cell sorting (Figure 2A). The bulk cultures from 3 different donors were enriched for IL-10 producing cells. The percentage of IL-10 producing T cells after enrichment ranged between 18% and 93%. T-cell clones were isolated from the enriched cultures using limiting dilution.

The isolated T-cell clones displayed a CD3+CD4+CD8- phenotype and were assessed for clonality by analysis of their TCR-Vβ using flow cytometry. In total 125 M1-specific T-cell clones were isolated. Consistent with the cultures at the bulk level, the isolated influenza specific T-cell clones recognized naturally processed M1 protein (Figure 2C). Moreover, the clones specifically proliferated when stimulated with live virus infected monocytes, as one would expect from

influenza-specific CD4+ T cells (Figure 2E). Few clones did not respond to viral challenge, and is likely due to differences in amino acid sequence between the synthetic M1 peptides (based on A/PR/8/34) and the virus used (A/Wisconsin/67/2005).

The M1-specific T-cell clones had a diverse phenotype in terms of IL-10 and IFNγ production. Forty-two of the M1 specific clones did not produce IL-10 upon antigen challenge, which could be explained by the fact that the T-cell clones were isolated from IL-10 enriched, but not pure populations. Consistent with findings in mice (20), most of the IL-10 producing clones (79/83) produced both IFNγ and IL-10 upon cognate peptide stimulation (Figure 2BD). Analysis of the clones on a single cell level using cytokine capture assay revealed that the same cell produced both IFNγ and IL-10 at high concentrations of cognate peptide. However, in some cases (D4.6 and D4.11) T-cell clones produced only IL-10 in the lower antigen-range, but co-produced IFNγ when stimulated with increasing concentrations of M1 peptide (Figure 3). Other clones (e.g. D4.18; Figure 3) produced only IFNγ upon peptide stimulation.

Subsequently, the expression of FOXP3 in these clones was examined. Previously we showed that non-suppressive T-cell clones display an increased expression of FOXP3 compared to naïve T cells analyzed by flow cytometry but lack intra-nuclear localization, and that the level of FOXP3 was much higher in Tregs (10, 14). Therefore, the levels of FOXP3 were compared to that of established FOXP3-positive and FOXP3-negative clones (C148.31 and C271.9 respectively). In total 16 of the 59 randomly selected clones (28%) showed a considerable level of constitutive FOXP3 expression, as determined 21 days after last antigen-specific activation (Figure 2F).

M1-specific T cells with antigen-dependent suppressive capacity are predominantly present within the IL-10 producing population. The cytokine profile of the influenza-specific CD4+ T-cell clones resemble that of Tregs specific for chronic infections and tumor antigens (14, 16, 27). Therefore, the isolated clones were further expanded and tested for their suppressive capacity. In total, 26 of 69 clones that could be tested showed significant suppression (>50%) on the proliferation of anti-CD3 stimulated CD4+CD25- T cells. Division of the T-cell clones based on IL-10 production, IL10- (<50 pg/ml) and IL-10+ (>50 pg/ml), revealed that the Tregs are significantly more found among the population of IL-10 producing T cells ($p<0.001$; two tailed Mann-Whitney test), but are not exclusively found within this population (Figure 4A). A number of influenza-specific CD4+ Treg clones were studied in more detail. These suppressive clones not only prevented proliferation of CD3-stimulated effector cells, but also their capacity to produce IFNγ (Figure 4B).

To study whether these clones could also exert their suppressive function when activated through their TCR upon recognition of cognate antigen, the Treg clones were stimulated with M1 peptide during the assay (Figure 4C). CFSE-labeled responder cells were stimulated with allogeneic APC in the presence of a PKH-26 labeled influenza-specific T-cell clone (Figure 4 C; upper panels). Consistent with the anti-CD3 based suppression assay, the clones D1.6, D1.52, D4.6, and D1.68 were able to suppress proliferation upon stimulation with M1 peptide, whereas D1.50 and D4.11 did not suppress proliferation (Figure 4C; lower panels). Notably, the amount of Tregs in the bulk culture was insufficient to induce overt suppression (See example in Figure 4D). These data indicate that influenza-specific Tregs are present in healthy donors, but the Tregs do not dominate the M1-

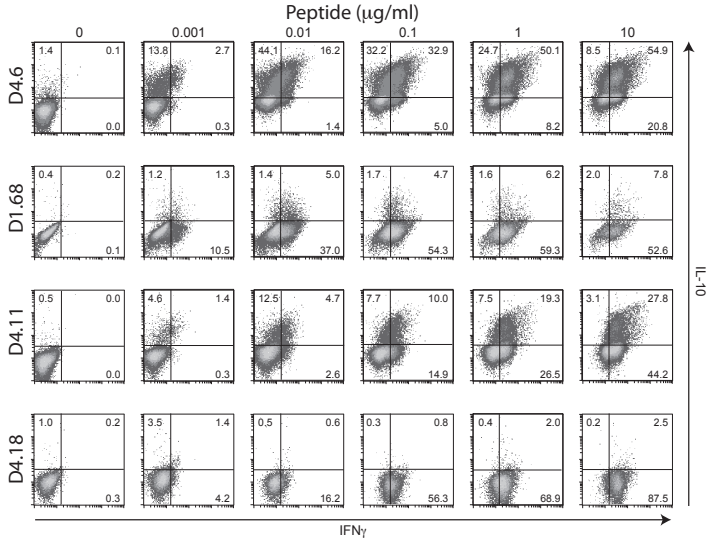

Figure 3. IL-10 and IFNγ production by M1-specific T-clones at the single cell level. M1-specific production of IL-10 and IFNγ following stimulation with APC pulsed with increasing concentrations of M1 peptide was analyzed on a single cell level by cytokine capture assay. The percentage of cytokine producing cells is indicated in the corners of the plots. D4.18, which is presented as control clone, recognized M1 peptide 196-225.

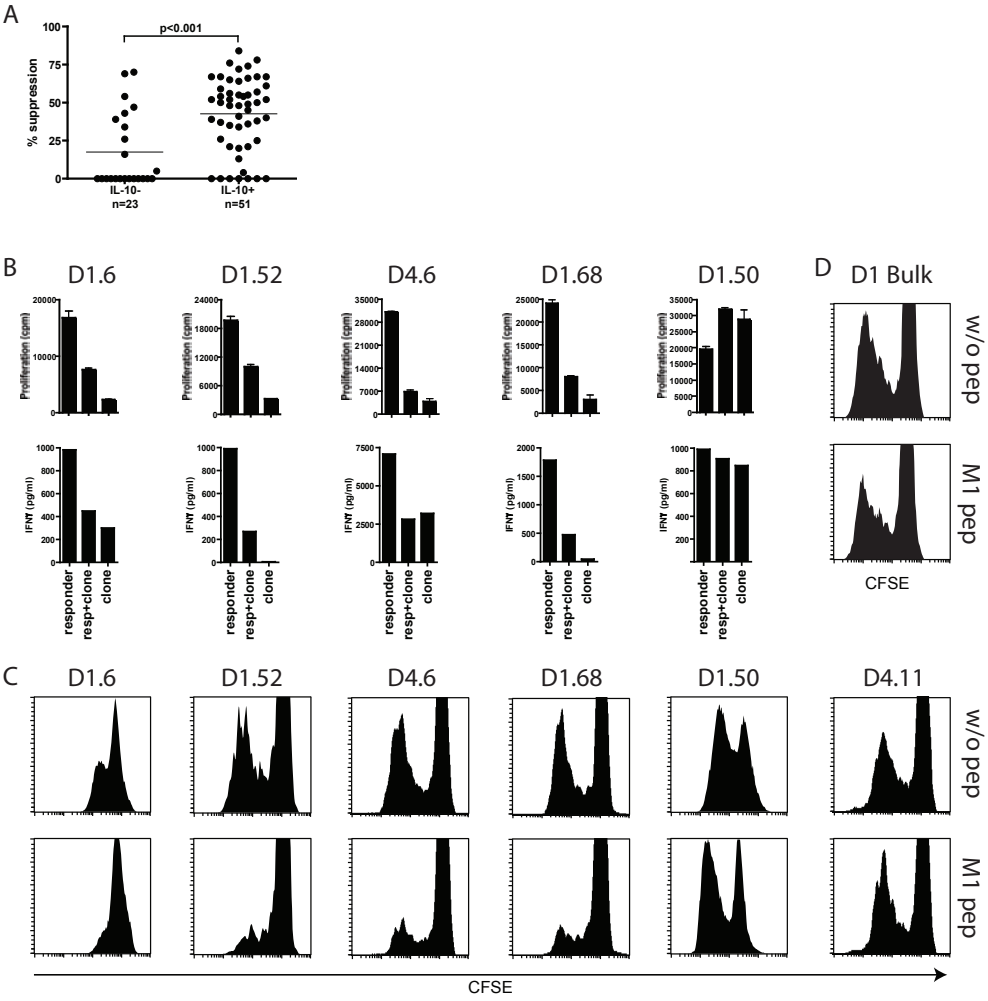

Figure 4. IL-10 producing M1-specific T-cell clones comprise Treg which suppress in an antigen dependent manner. A) Suppressive capacity of M1-specific clones on proliferation in anti-CD3 dependent suppression assay. Responder cells were co-cultured with M1-specific T-cell clones at a 1:1 ratio and compared to proliferation of responder cells only to calculate the percentage of suppression. The M1-specific clones isolated from 3 donors were divided in 2 groups dependent on IL-10 production (IL-10-: <50 pg/ml and IL-10+: >50 pg/ml). B) Suppressive capacity of individual clones on proliferation (top panels) and IFNγ production (bottom panels) in anti-CD3 dependent suppression assay. The responders and clones were co-cultured at a 1:1 ratio. C) Antigen-dependent suppression of IL-10 producing T-cell clones. CFSE-labeled responder cells were co-cultured with M1-specific T-cell clones at 1:1 ratio and stimulated with allogeneic APC. In the upper panels only the responder cells are stimulated, in the lower panels the M1-specific T-cell clone is stimulated as well with cognate peptide. The proliferation of the CFSE-labeled responder cells is presented. D) Antigen dependent suppression of D1 T-cell bulk culture as described in (C).

specific T-cell population expanded from PBMC in vitro.

In order to test whether the Treg clones could also suppress when their cognate antigens are present in the natural context, we tested the suppressive capacity of D1.68 when stimulated by APC infected with live influenza virus (Figure 5). Importantly, the proliferation of the responder cells was not influenced by the presence of influenza virus (Figure 5A; upper panels and Figure 5B left set of columns). Simply adding the Treg clone B1.68 did not result in substantial suppression of the responder cells either. However, in the presence of influenza virus-infected antigen presenting cells D1.68 Treg cells were activated and able to suppress the proliferation of the responder cells in a dose-dependent manner (Figure 5A; middle panels and Figure 5B middle set of columns). As a control, the non-suppressive T-cell clone D1.50 was added, but this clone was not able to suppress the responder cells. These data indicate that the influenza-specific Tregs are able to suppress other T cells under conditions that resemble influenza infection.

Treg clones interfere with the IL-2 pathway at multiple levels. IL-2 production by T-helper cells plays a critical role in the induction and sustainment of CTL (28) and can be suppressed by Treg (14, 27). To assess whether IL-2 production by influenza-specific T-helper cells was inhibited by influenza-specific Treg, a co-culture experiment was performed wherein the CFSE-labeled T-helper clone D1.50 started to produce IL-2 when APC presented the clone's cognate antigen. Upon stimulation of the Treg clone (either FOXP3+ or FOXP3-), already present in the co-culture, the production of IL-2 by D1.50 was inhibited (Figure 6A). This shows that IL-2 production by influenza-specific T-helper cells is inhibited by Treg cells specific for the same viral antigen.

Quickly after activation CD8+ T cells start to upregulate the high-affinity chain of the IL-2 receptor (CD25) at their cell surface as this is critical for maintaining the CD8 T-cell response (28). To study the effect of Tregs on the expression of the IL-2 receptor by CD8 T cells, OKT-3 dependent upregulation of CD25 by CD8+ PBMC in the absence or presence of M1-specific Treg was examined. Additionally, upregulation of CD69, which is a very early activation marker with unknown function, and 4-1BB (CD137), which is important for T-cell survival (29) was analyzed. Stimulated CD8+ PBMC upregulated CD25, CD69 and CD137 (Figure 6B) but when the CD8+ PBMC were activated in the presence of M1-specific Treg clones B1.6 or B1.52, the upregulation of CD25 was inhibited while both CD69 and CD137 were still upregulated. This indicates that the CD8 are partly activated in the presence of Treg, but are incapacitated to ingest IL-2 required for their full expansion, consistent with the data previously reported in murine models (30). As a control, there was no effect on CD25 upregulation when the CD8+ PBMC were cocultured with M1-specific bulk culture. These data imply that the M1-specific Treg specifically interfere with the IL-2 pathway on multiple levels, both on the production by T-helper cells as well as IL-2 uptake by CD8+ effector cells.

Discussion

In this study we showed that the influenza M1-specific proliferative T-cell response is accompanied by the production of both IFNγ and IL-10, similar to earlier observations in a mouse model (20). In-depth analysis of this immune response at the T-cell clonal level revealed that M1-specific T cell could simultaneously produce IL-10 and IFNγ. Furthermore, a substantial proportion of the IL-10 producing M1-specific T-cell clones exerted a suppressive capacity when stimulated with their cognate peptide-antigen. The population of Treg clones comprised both FOXP3- and FOXP3+ T-cell clones, consistent with the previously reported population of HPV-specific Treg (14). Moreover, the M1-specific Tregs were able to suppress the proliferation, IL-2 and IFNγ autologous T-helper type 1 cells in an antigen-dependent manner as well as able to suppress the full expansion of CD8 T cells by inhibiting the upregulation of the high affinity IL-2 receptor on activated CD8+ T cells. These data clearly show that influenza-specific Treg cells are induced in healthy donors as a consequence of previous infection with influenza virus.

Previously, we have shown that virus-specific Tregs could be isolated from patients suffering from human papilloma virus-induced lesions (14, 17). The absence of sufficient concentrations of live HPV virus prohibited us to study the suppressive function of the HPV-specific Treg when their antigen was presented in the natural context. Fortunately, influenza virus is readily available and allowed us to use influenza-infected APC to stimulate M1-specific Treg in order to show that they were able to suppress the proliferation of effector cells. Indeed our current study shows that pathogen-specific Treg are fully capable of exerting their effector function when stimulated with

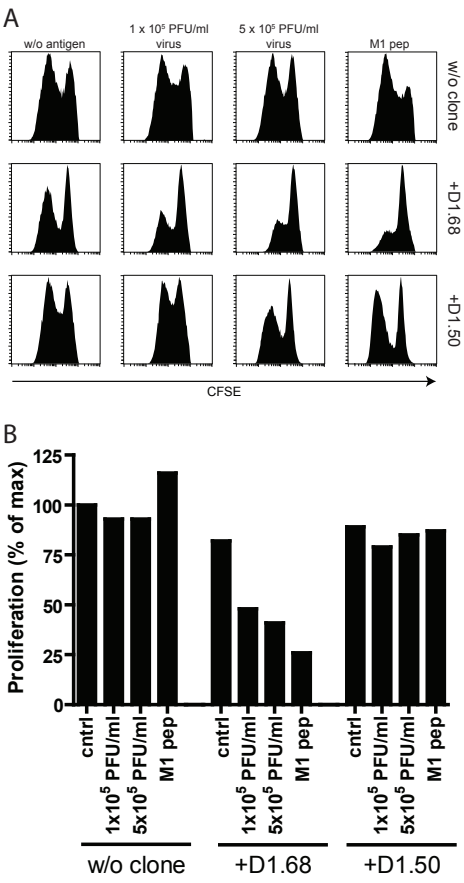

Figure 5. Treg clones are able to exert their suppressive function after recognition of influenza virus–infected APC. Antigen–dependent suppression of IL–10 producing T–cell clones. APC were infected with influenza virus. Treg clone D1.68 was stimulated with APC infected with increasing amounts of influenza virus. D1.50 without suppressive capacity is shown as control. A) Proliferation of the CFSE–labeled responder cells is presented. B) The proliferation of the responder cells was calculated using the FlowJo software. Proliferation of the condition without clone and antigen was set to 100%.

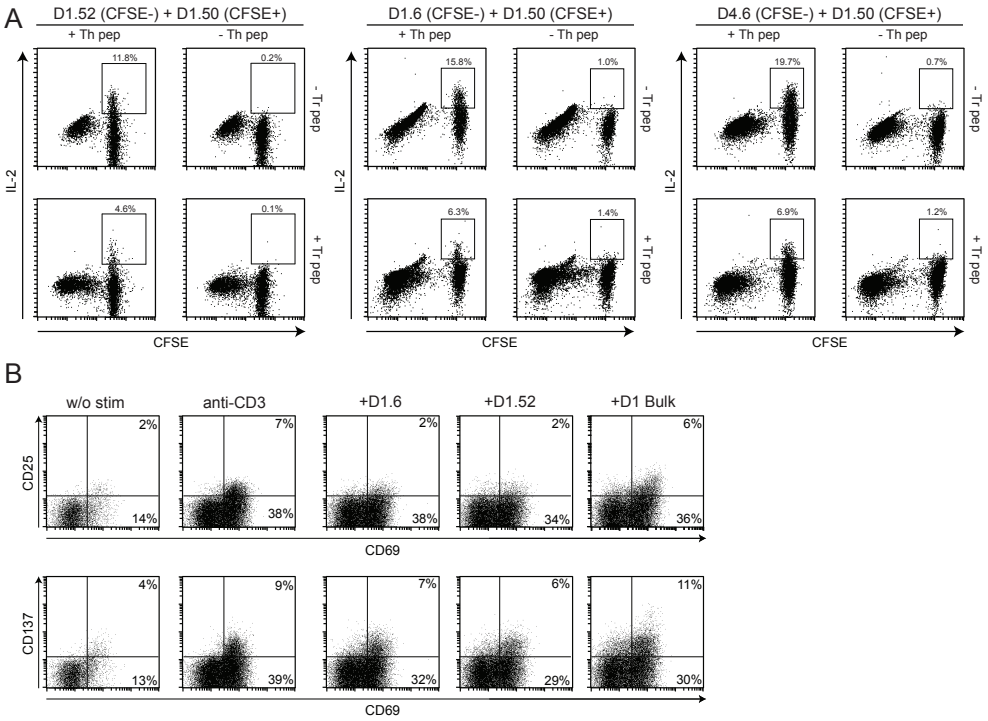

Figure 6. M1-specific Treg interfere with the IL-2 pathway at multiple levels. A) Suppression of IL-2 production (by T-helper clone D1.50) by 2 FOXP3+ and 1 FOXP3- M1-specific clones in an autologous setting. Where indicated, 5µg/ml of M1 peptide cognate to CFSE-labeled D1.50 (Th pep) and/or 5 µg/ml M1 peptide cognate to Treg clone (Tr pep) was added to the cultures. The percentage of IL-2 producing D1.50 is indicated. B) Suppression of IL-2 receptor upregulation by activated CD8+ responder cells. Freshly isolated CD8 T cells were stimulated with agonistic anti-CD3 antibody in the presence or absence of Treg clone or D1 bulk T-cell culture as control. Upregulation of the with T-cell activation associated markers CD25 (IL-2R α-chain), CD69 and CD137 was analyzed 24 hours after stimulation. The percentage of CD8+ T cells expressing each of the activation markers is shown.

influenza-infected APC resembling the natural context in which these T cells would detect their cognate antigen in vivo.

There are numerous studies reporting pathogen-specific Tregs in chronic infections (14, 16, 17, 19). To our knowledge, the question whether pathogen-specific Tregs are also induced under acute non-pathogenic viral infections in humans has not been adequately addressed. Since influenza infections are characterized by acute onset and lack a chronic phase (2), our data reveals that virus-specific Tregs are also induced as a result of acute viral infections.

Highly pathogenic influenza infections including H5N1 and the 1918 influenza virus are characterized by a cytokine storm which contributes to the lethality of these viruses (31-33). The observed cytokine storm includes several proinflammatory cytokines and chemokines which are also increased after IL-10 blockade during sublethal influenza infection (34). In people infected with dengue virus, which causes immunopathology similar to high pathogenic influenza, it was found that a high Treg/Teffector ratio correlated with a mild disease (35), indicating immunopathology can develop as a result of insufficient expansion of Treg during acute infection. This raises the question whether influenza-specific Treg are insufficiently expanded during infection with highly pathogenic influenza viruses. Notably, under conditions where influenza infection is controlled, the population of IL-10 producing CD4+ T cells is activated early during influenza infection in order to peak 2-3 days after the virus is cleared from the lung (3). One of the mechanisms involved is likely to be the reduction of IL-2 producing cells as well as prevention of IL-2 receptor upregulation (Figure 6), together playing a critical role in the sustainment of the influenza-specific CD4 and CD8 effector cell subsets (36), which are known to produce the different types of proinflammatory cytokines found during influenza infection (3).

In summary, we showed that pathogen-specific IL-10 producing Treg are induced during acute virus infections. These Tregs are potentially important in preventing immunpathology which may occur otherwise as a result of an uncontrolled anti-viral immune response.

Acknowledgements

This study was financially supported by a grant from the Netherlands Organization for Scientific Research (Zon/Mw 917.56.311 to S.H.v.d.B.). We would like to thank

Klara Broadway for technical assistance.

References

1. Wong, S.S.Y., and K. Yuen. 2006. Avian influenza virus infections in humans. Chest 129:156-168.
2. Rothberg, M.B., S.D. Haessler, and R.B. Brown. 2008. Complications of viral influenza. Am J Med 121:258-264.
3. Carding, S.R., W. Allan, A. McMickle, and P.C. Doherty. 1993. Activation of cytokine genes in T cells during primary and secondary murine influenza pneumonia. J Exp Med 177:475-482.
4. Moore, K.W., R. de Waal Malefyt, R.L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 19:683-765.
5. Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. Cell 75:263-274.
6. Gazzinelli, R.T., M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kuhn, W. Muller, G. Trinchieri, and A. Sher. 1996. In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. J Immunol 157:798-805.
7. O'Garra, A., and P. Vieira. 2007. T(H)1 cells control themselves by producing interleukin-10. Nat Rev Immunol 7:425-428.
8. Roers, A., L. Siewe, E. Strittmatter, M. Deckert, D. Schluter, W. Stenzel, A.D. Gruber, T. Krieg, K. Rajewsky, and W. Muller. 2004. T cell-specific inactivation of the interleukin 10 gene in mice results in enhanced T cell responses but normal innate responses to lipopolysaccharide or skin irritation. J Exp Med 200:1289-1297.
9. Rubtsov, Y.P., J.P. Rasmussen, E.Y. Chi, J. Fontenot, L. Castelli, X. Ye, P. Treuting, L. Siewe, A. Roers, W.R. Henderson, Jr., W. Muller, and A.Y. Rudensky. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity 28:546-558.
10. Piersma, S.J., M.J. Welters, and S.H. van der Burg. 2008. Tumor-specific regulatory T cells in cancer patients. Hum Immunol 69:241-249.
11. Kretschmer, K., I. Apostolou, D. Hawiger, K. Khazaie, M.C. Nussenzweig, and H. von Boehmer. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. Nat Immunol 6:1219-1227.
12. Zhou, G., C.G. Drake, and H.I. Levitsky. 2006. Amplification of tumor-specific regulatory T cells following therapeutic cancer vaccines. Blood 107:628-636.
13. Curotto de Lafaille, M.A., N. Kutchukhidze, S. Shen, Y. Ding, H. Yee, and J.J. Lafaille. 2008. Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation. Immunity 29:114-126.
14. van der Burg, S.H., S.J. Piersma, A. de Jong, J.M. van der Hulst, K.M. Kwappenberg, M. van den Hende, M.J. Welters, J.J. Van Rood, G.J. Fleuren, C.J. Melief, G.G. Kenter, and R. Offringa. 2007. Association of cervical cancer with the presence of CD4+ regulatory T cells specific for human papillomavirus antigens. Proc Natl Acad Sci U S A
15. Suffia, I.J., S.K. Reckling, C.A. Piccirillo, R.S. Goldszmid, and Y. Belkaid. 2006. Infected site-restricted Foxp3+ natural regulatory T cells are specific for microbial antigens. J Exp Med 203:777-788.
16. Ebinuma, H., N. Nakamoto, Y. Li, D.A. Price, E. Gostick, B.L. Levine, J. Tobias, W.W. Kwok, and K.M. Chang. 2008. Identification and in vitro expansion of functional antigen-specific CD25+ FoxP3+ regulatory T cells in hepatitis C virus infection. J Virol 82:5043-5053.
17. de Vos van Steenwijk, P.J., S.J. Piersma, M.J. Welters, J.M. van der Hulst, G. Fleuren, B.W. Hellebrekers, G.G. Kenter, and S.H. van der Burg. 2008. Surgery followed by persistence of high-grade squamous intraepithelial lesions is associated with the induction of a dysfunctional HPV16-specific T-cell response. Clin Cancer Res 14:7188-7195.
18. Belkaid, Y. 2007. Regulatory T cells and infection: a dangerous necessity. Nat Rev Immunol 7:875-888.
19. Rouse, B.T., P.P. Sarangi, and S. Suvas. 2006. Regulatory T cells in virus infections. Immunol Rev 212:272-286.
20. Debes, G.F., M.E. Dahl, A.J. Mahiny, K. Bonhagen, D.J. Campbell, K. Siegmund, K.J. Erb, D.B. Lewis, T. Kamradt, and A. Hamann. 2006. Chemotactic responses of IL-4-, IL-10-, and IFN-gamma-producing CD4+ T cells depend on tissue origin and microbial stimulus. J Immunol 176:557-566.
21. van der Burg, S.H., M.E. Ressing, K.M. Kwappenberg, A. de Jong, K. Straathof, J. de Jong, A. Geluk, K.E. van Meijgaarden, K.L. Franken, T.H. Ottenhoff, G.J. Fleuren, G. Kenter, C.J. Melief, and R. Offringa. 2001. Natural T-helper immunity against human papillomavirus type 16 (HPV16) E7-derived peptide epitopes in patients with HPV16-positive cervical lesions: identification of 3 human leukocyte antigen class II-restricted epitopes. Int J Cancer 91:612-618.
22. Piersma, S.J., M.J. Welters, J.M. van der Hulst, J.N. Kloth, K.M. Kwappenberg, B.J. Trimbos, C.J. Melief, B.W. Hellebrekers, G.J. Fleuren, G.G. Kenter, R. Offringa, and S.H. van der Burg. 2008. Human papilloma virus specific T cells infiltrating cervical cancer and draining lymph nodes show remarkably frequent use of HLA-DQ and -DP as a restriction element. Int J Cancer 122:486-494.
23. Welters, M.J.P., G.G. Kenter, S.J. Piersma, A.P.G. Vloon, M.J.G. Löwik, T.M.A. Berends-van der Meer, J.W. Drijfhout, A.R.P.M. Valentijn, A.R. Wafelman, J. Osstendorp, G.J. Fleuren, R. Offringa, C.J.M. Melief, and S.H. van der Burg. 2008. Induction of Tumor-Speific CD4+ and CD8+ T-Cell Immunity in Cervical Cancer Patients by a Human Papillomavirus Type 16 E6 and E7 Long Peptides Vaccine. Clinical Cancer Research 14:
24. de Jong, A., J.M. van der Hulst, G.G. Kenter, J.W. Drijfhout, K.L. Franken, P. Vermeij, R. Offringa, S.H. van der Burg, and C.J. Melief. 2005. Rapid enrichment of human papillomavirus (HPV)-specific polyclonal T cell populations for adoptive immunotherapy of cervical cancer. Int J Cancer 114:274-282.
25. Welters, M.J., A. de Jong, S.J. van den Eeden, J.M. van der Hulst, K.M. Kwappenberg, S. Hassane, K.L. Franken, J.W. Drijfhout, G.J. Fleuren, G. Kenter, C.J. Melief, R. Offringa, and S.H. van der Burg. 2003. Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter. Cancer Res 63:636-641.
26. Welters, M.J., P. van der Logt, S.J. van den Eeden, K.M. Kwappenberg, J.W. Drijfhout, G.J. Fleuren, G.G. Kenter, C.J. Melief, S.H. van der Burg, and R. Offringa. 2006. Detection of human papillomavirus type 18 E6 and E7-specific CD4+ T-helper 1 immunity in relation to health versus disease. Int J Cancer 118:950-956.
27. Wang, H.Y., D.A. Lee, G. Peng, Z. Guo, Y. Li, Y. Kiniwa, E.M. Shevach, and R.F. Wang. 2004. Tumor-specific human CD4+ regulatory T cells and their ligands: implications for immunotherapy. Immunity 20:107-118.
28. Waldmann, T.A. 2006. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. Nat Rev Immunol 6:595-601.
29. Sabbagh, L., L.M. Snell, and T.H. Watts. 2007. TNF family ligands define niches for T cell memory. Trends Immunol 28:333-339.
30. Piccirillo, C.A., and E.M. Shevach. 2001. Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. J Immunol 167:1137-1140.
31. Cheung, C.Y., L.L. Poon, A.S. Lau, W. Luk, Y.L. Lau, K.F. Shortridge, S. Gordon, Y. Guan, and J.S. Peiris. 2002. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? Lancet 360:1831-1837.
32. de Jong, M.D., C.P. Simmons, T.T. Thanh, V.M. Hien, G.J. Smith, T.N. Chau, D.M. Hoang, N.V. Chau, T.H. Khanh, V.C. Dong, P.T. Qui, B.V. Cam, Q. Ha do, Y. Guan, J.S. Peiris, N.T. Chinh, T.T. Hien, and J. Farrar. 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat Med 12:1203-1207.
33. Kobasa, D., S.M. Jones, K. Shinya, J.C. Kash, J. Copps, H. Ebihara, Y. Hatta, J.H. Kim, P. Halfmann, M. Hatta, F. Feldmann, J.B. Alimonti, L. Fernando, Y. Li, M.G. Katze, H. Feldmann, and Y. Kawaoka. 2007. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. Nature 445:319-323.
34. Sun, J., R. Madan, C.L. Karp, and T.J. Braciale. 2009. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. Nat Med 15:277-284.
35. Luhn, K., C.P. Simmons, E. Moran, N.T. Dung, T.N. Chau, N.T. Quyen, T.T. Thao le, T. Van Ngoc, N.M. Dung, B. Wills, J. Farrar, A.J. McMichael, T. Dong, and S. Rowland-Jones. 2007. Increased frequencies of CD4+ CD25(high) regulatory T cells in acute dengue infection. J Exp Med 204:979-985.
36. Letourneau, S., C. Krieg, G. Pantaleo, and O. Boyman. 2009. IL-2- and CD25-dependent immunoregulatory mechanisms in the homeostasis of T-cell subsets. J Allergy Clin Immunol 123:758-762.