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Reduced circulating $CD4^+CD25^+$ cell populations in haemorrhagic fever with renal syndrome

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Summary

Immunopathological mechanisms are speculated to underlie haemorrhagic fever with renal syndrome (HFRS) caused by Hantaviruses. $CD4^+CD25^+$ T regulatory cells (T_{regs}), a subset of $CD4^+$ T cells, expressed high levels of CD25 and the forkhead box transcription factor P3 (FoxP3), plays an important role in the down-regulation of various immune responses. Therefore, we hypothesized that in patients with HFRS the immunopathology could be, at least in part, the result of an inefficient control of pathogenic effector T cells by T_{regs}. The number of T_{regs} was determined by flow cytometry according to their characteristic $CD4^+CD25^{high}$ membrane phenotype. The functional characterization of T_{regs} was analysed by suppression of proliferation and secretion of cytokines by co-cultured effector $CD4^+CD25^-$ T cells. FoxP3 mRNA level was assessed by quantitative real-time polymerase chain reaction. We observed that $CD4^+CD25^{high}$ cells of patients with HFRS showed a conventional phenotype. Furthermore, acute-stage patients with HFRS exhibited significantly reduced numbers of peripheral T_{regs} compared with healthy donors, and marked improvement was observed in convalescent-phase patients. The frequency of T_{regs} was correlated positively with platelet count, and was correlated negatively with blood urea nitrogen, serum creatinine and serum aspartate aminotransferase. On the other hand, T_{regs} from both healthy individuals and patients with HFRS exhibited equal FoxP3 expression of mRNA, and their ability to suppress the proliferation and cytokine secretion of $CD4^+$ effector T cells was unimpaired in HFRS patients.

Keywords: Hantavirus, immune regulation, T regulatory cells

Introduction

$CD4^+CD25^+$ T regulatory cells (T_{regs}), described as a minor population of $CD4^+$ T cells, express high levels of CD25, play an important role in the control of immune reactivity against self-antigens and have the ability to inhibit chronic inflammatory responses [1,2]. Although studies have shown clearly that T_{regs} are produced mainly in the thymus and present in healthy individuals from birth, these cells can also be derived from mature naive $CD4^+CD25^-$ T cells in the periphery under special stimulatory conditions [3–5]. The T_{regs} subset express constitutively a variety of cell surface molecules, such as CD25, $CD45RB^{low}$, CD62L and CTLA-4, as well as glucocorticoid-induced tumour necrosis factor receptor.

Glucocorticoid-induced tumour necrosis factor receptor associated more commonly with activated/memory cells [6–8]. The forkhead box/winged helix transcription factor P3 (FoxP3) has been identified as a key regulatory gene for the development and function of T_{regs} [9]. Spontaneous mutation of FoxP3 leads to widespread lymphocytosis and autoimmunity in mice and humans with immune deregulation, polyendocrinopathy, enteropathy and X-linked syndrome [10,11]. Although T_{regs} require activation by antigen exposure to initiate suppressive functions, the effector (suppression) phase is independent of antigen specificity [12,13]. Soluble cytokines and cell–cell contact-dependent mechanisms have been shown to contribute to the suppressive activities mediated by T_{regs} [14,15]. In humans, an alteration in the generation and development of the suppressive function of T_{regs} is often associated with autoimmune diseases [16–18].

Haemorrhagic fever with renal syndrome (HFRS), characterized by altered vascular permeability and acute thrombocytopenia, is a zoonosis caused by different species of

Hantaviruses. More than 100 000 cases of HFRS are reported annually in Asia and Europe and most are reported in China [19]. Hantaviruses infect predominantly endothelial cells and macrophages, but infection has no direct cytopathic effect on these or other cells [20–22], indicating that direct viral cytotoxicity is not responsible for the pathology observed in humans. Several findings suggest that patients with HFRS are in a state of high-level cellular immune response, which may be involved in the development of inflammation and pathological lesions [23,24]. For example, in the early stages of HFRS, decreased activity of spontaneous suppressor T cells was concurrent with increased numbers of $CD8^+$ cells and a reversed CD4 : CD8 ratio [25,26]. The strong Hantavirus-specific $CD8^+$ T cells responses might be responsible for the damage of epithelial cell apoptosis during HFRS/Hantavirus pulmonary syndrome (HPS), which caused elevated levels of extracellular perforin and granzyme B [27,28]. Moreover, infection of dendritic cells with Hantavirus induced the release of the proinflammatory cytokines tumour necrosis factor-α and interferon (IFN)-α [29]. So far, there are still no studies of T_{regs} in HFRS patients. Only one study showed that the ratio of activated antigen (CD25)-positive lymphocytes of peripheral blood mononuclear cells (PBMC) in the acute phase of HFRS was higher than that in convalescent phase [26]. The finding has implied the possibility that T_{regs} play a role in the development of inflammation and pathological lesions.

In order to elucidate the role of T_{regs} in the pathogenesis of HFRS, we measured the percentage of T_{regs} in HFRS patients and investigated their suppressive activities in inhibiting proliferation and cytokine secretion of $CD4^+$ effector T cells. Our data showed that T_{regs} numbers were reduced, but their suppressive function was intact during the acute stage of HFRS. In addition, marked improvement in the number of T_{regs} was observed in convalescent patients.

Materials and methods

Patients and specimens

In this study, 76 individuals with HFRS (24 females and 32 males, age 20–50 years, mean age 40 $\pm$ 11·0 years) were analysed. All patients were confirmed serologically HFRS by enzyme-linked immunosorbent assay (ELISA) and/or an immunofluorescence test for specific immunoglobulin (Ig)M and IgG. The patients were hospitalized in the acute phase of illness, between 2 and 12 days (mean 8 days) after the onset of HFRS. We obtained medical records of clinical symptoms and signs and the following laboratory values from each patient with HFRS; haemoglobin, haematocrit, platelet count, leucocyte count, blood urea nitrogen (BUN), serum creatinine, serum albumin level, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) levels, proteinuria and 24-h urine protein amount. Hypotension was defined as systolic pressure below 90 mmHg. Thrombocytopenia was defined as a platelet count below 100 000/l. Acute renal failure was defined as a serum creatinine over 2 mg/dl. Abnormal liver function was defined as an AST or ALT over 80 IU/l. Forty-seven patients were in febrile, hypotensive or oliguric phase, and 29 entered polyuric phase. Abnormal liver function developed in 54 (71·1%) patients. Haemorrhage manifestation developed in 28 (36·8%) patients. Laboratory findings at admission are listed in Table 1.

Table 1. Laboratory findings in 76 patients with haemorrhagic fever with renal syndrome (HFRS) at admission.

	Mean $\pm$ s.d.	Range	Reference values
Hb (g/dl)	12·1 $\pm$ 2·7	8·1–21·9	12–16
Hct (%)	41·5 $\pm$ 6·4	24·1–62·8	34–49
WBC ($\times 10^3$/l)	14·7 $\pm$ 10·9	3·8–58·2	5–10
Plt ($\times 10^3$/l)	71 $\pm$ 62	10–314	150–450
BUN (mg/dl)	57·8 $\pm$ 36·1	5·1–101·9	8–23
Scr (mg/dl)	5·6 $\pm$ 3·7	0·7–12·1	0·5–1·2
AST (IU/l)	155 $\pm$ 102	18–870	13–36
ALT (IU/l)	74 $\pm$ 79	11–414	5–33

Hb, haemoglobin; Hct, haematocrit; WBC, white blood cell; Plt, platelet; BUN, blood urea nitrogen; Scr, serum creatinine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; s.d., standard deviation.

Acute blood samples were drawn at the time of hospitalization. Convalescent-phase samples were drawn 1 month after recovery from the disease. Thirty healthy donors (10 females and 20 males, age 18–52 years, mean age 40 $\pm$ 1·2 years) were included. Ethical approval was obtained and informed consent was obtained from all patients.

Flow cytometry analysis

Three-colour flow cytometry analysis was performed to quantify T_{regs} in human peripheral blood. For immunostaining, phycoerythrin, fluorescein isothiocyanate and peridinin chlorophyll protein conjugated monoclonal antibodies against CD4 (clone: L200), CD25 (clone: M-A251), FoxP3 (clone: 206D), CTLA-4 (clone: BNI3), CD45RO (clone: UCHL1), CD62L (clone: IM1231), CD69 (clone: CH-4) and CD95 (clone: DX2) were purchased from BD Biosciences (La Jolla, CA, USA). All antibodies were used at concentrations titrated for optimal staining. Briefly, a sample of peripheral blood was incubated in the dark for 30 min, washed with phosphate-buffered saline (PBS) twice and analysed in a FACSCalibur (Becton Dickinson). Intracellular staining of CTLA-4 and FoxP3 was performed by using fixation and permeabilization buffers according to the manufacturer's instructions, followed by visualization with streptavidin antibody. Suitable isotype controls were performed. To estimate the absolute numbers of T_{regs}, 200 µl of CD4- and CD25-labelled blood were added to 800 µl sheath fluid and 100 µl of FlowCount Fluorospheres (Beckman/Coulter Inc., Fullerton, CA, USA) counting beads and vortexed gently for 20 s. The

number of cells in the chosen gate was assessed by counting the number of events in the gate on two occasions, taking the average value and then multiplying by the number of counting spheres/μl and the dilution factor. Flow cytometric analysis was performed on a FACSCalibur cytometer. Data processing was accomplished with CELLQuest software (Becton Dickinson).

Isolation and purification of T cell subsets

The PBMCs were separated by Ficoll density centrifugation. $CD4^+$ T cells were isolated through negative selection by removing all other cell types after 15-min incubation with a mixture of biotin-conjugated antibodies. The resulting population was, on average, 95% and $CD4^+$ was separated further into $CD25^+$ and $CD25^-$ fractions by CD25 microbeads (Miltenyi Biotec, Milburn, CA, USA). A modified version of the protocol for positive selection was used to separate $CD4^+CD25^{high}$ cells that have been described as highly enriched in T_{regs} [30]; $CD4^+$ cells were incubated first with 5 μl of CD25 beads/10^7 cells and underwent three consecutive simple positive selections (autoMACS; Miltenyi Biotec) to obtain the $CD25^{high}$ population. The initial negative fraction was reincubated with CD25 beads at 20 μl/10^7 cells and underwent 'sensitive depletion' (an autoMAC option) to obtain the $CD25^-$ subset. The purity of the separated $CD4^+CD25^{high}$ cells and $CD4^+CD25^-$ cells subsets was confirmed by flow cytometry.

Real-time quantitative polymerase chain reaction

The FoxP3 mRNA expression was quantified by real-time polymerase chain reaction (PCR) using ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). The human housekeeping gene β-actin primers and probe set was used as a reference for sample normalization. Total RNA isolated from $CD4^+CD25^{high}$ T cell was reverse-transcribed into cDNA by using primed random hexamer. The primer set for FoxP3 was 5′-TTCGAAGAGCCAG AGGACTT-3′ and 5′-GCTGCTCCAGAGACTGTACC-3′. The probe for FoxP3 was 5′-FAM-CTCAAGCACTGCCA GGCGGACCATC-TAMRA-3′. The primer set for β-actin was 5′-ATCTGCTGGAAGGTGGACAGCGA-3′ and 5′-CCC AGCACAATGAAGATCAAGATCAT-3′. The probe for β-actin was 5′-FAM-TGAGCGCA AGTACTCCGTGTGGA TCGGCG-TAMRA-3′. The primers and probes used in the real-time PCR were ordered from Sangon (Shanghai, China) and designed not to amplify genomic DNA. Standard curves were generated from serial dilutions of purified plasmid DNA encoding the respective genes with a linear regression *R* greater than 0·99 and used to quantify mRNA copy numbers for each sample. The amplification protocol used was described as follows: 1 μl of synthesized cDNA product was added subsequently into PCR mix containing 25 μl of *Taq*Man 2× PCR master mix (Applied Biosystems), 30 pmol human FoxP3 primer with 10 pmol probe and 2·5 μl β-actin primer/probe set, and distilled water was added to make a total reaction volume of 50 μl. The PCR was programmed as an initial incubation for 10 min at 95°C followed by 40 thermal cycles of 15 s at 95°C and 1 min at 60°C. The normalized values in each sample were calculated as the relative quantity of FoxP3 mRNA expression divided by the relative quantity of β-actin mRNA expression. All reactions were confirmed by at least one additional independent run.

Proliferation assay

To assess the proliferative response of purified $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells, co-cultures were established in 96-well U-bottomed plates incubated with 0·5 μg/ml anti-CD3 monoclonal antibody (clone: UCHT1) overnight at 4°C, and washed. $CD4^+CD25^-$ T cells (responders) and $CD4^+CD25^{high}$ T cells (suppressor) (10^4 cells/well) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) in different responder/suppressor ratios (0:1, 1:1, 1:1/2, 1:1/4, 1:1/8, 1:0). To every well, $1{\cdot}0 \times 10^4$ irradiated (2500 rads) PBMC were added as antigen-presenting cells and all cells were cultured in a final volume of 200 μl. All tests were conducted in triplicate. Cell cultures were then incubated at 37°C for 4 days and supernatants were obtained for cytokine measurements before being pulsed with 1 μCi [^{3}H]-thymidine per well for the final 18 h of incubation. Plates were harvested onto nylon filters using the Betaplate system and radioactivity was quantified using a Betaplate counter (Beckman Instruments, Fullerton, CA, USA). Results are expressed in counts per minute (cpm) as the mean of triplicate cultures ± standard deviation. Percentage suppression was calculated using the formula: (1 − cpm in presence of $CD4^+CD25^+$ T cells/cpm in absence of $CD4^+CD25^+$ T cells) × 100%.

Measurement of cytokine production

The supernatants that were removed before addition of [^{3}H]-thymidine were diluted for measurement of cytokine concentration by ELISA (R&D kits, Minneapolis, MN, USA). Briefly, microtitre plates pre-coated with capturing monoclonal antibodies were blocked with 2% bovine serum albumin (BSA)/PBS. After washing, samples and controls were added at 50 μl per well and incubated for 2 h with a biotinylated detecting antibody (50 μl per well) in 2% BSA/PBS/Tween-20. Plates were washed and incubated for 30 min with streptavidin-conjugated horseradish peroxidase. Next, 100 μl of 0·0125% tetramethylbenzidine and 0·008% H_2O_2 in citrate buffer was used as substrate. A standard curve was performed for each plate and used to calculate the absolute concentrations of cytokines.

Statistical analysis

Normally distributed data sets were analysed by Student's *t*-test, paired *t*-test, analysis of variance and linear regression

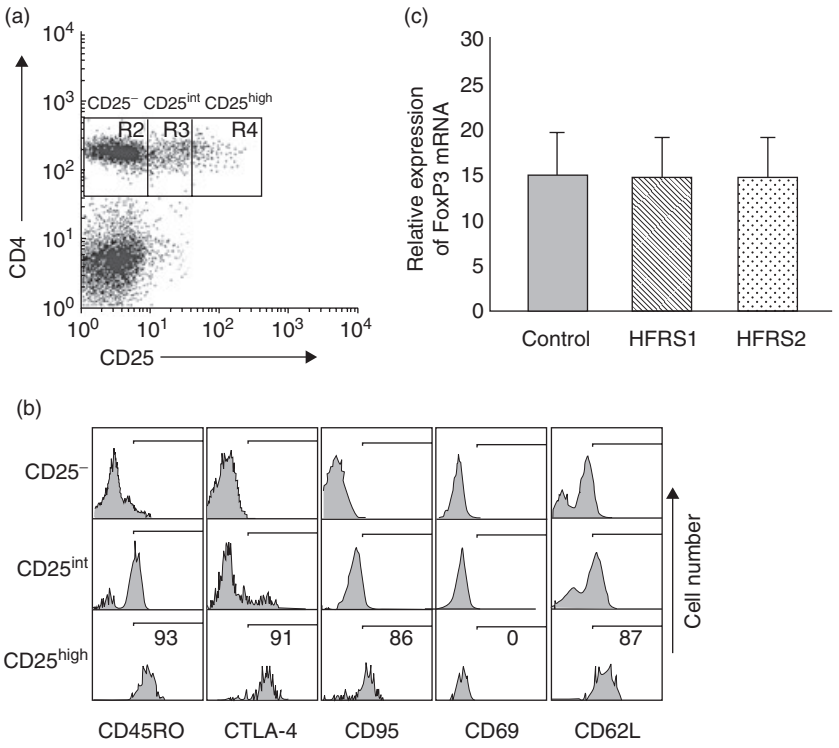

Fig. 1. Defining the human T regulatory cell (T_{reg}) population. (a) T_{regs} were identified as $CD4^+CD25^{high}$ T cells by selecting those CD4-positive cells whose CD25 expression exceeded the level of CD25 positivity seen on the CD4-negative population. (b) $CD4^+CD25^{high}$ T cells from either healthy subjects or patients expressed the same levels of CTLA4, CD45RO, CD95 or CD62L; CD69 was not expressed. Results are representative of 20 separate experiments performed on patients with haemorrhagic fever with renal syndrome (HFRS) and healthy subjects. (c) $CD4^+CD25^{high}$ T cells isolated from HFRS1 ($n = 20$), HFRS2 ($n = 20$) and healthy controls ($n = 20$) exhibited equal forkhead box P3 (FoxP3)-expression levels.

and correlation analysis (using Primer for Biostatistics). The Wilcoxon two-sample test and Kruskall–Wallis test were used for data sets that were not distributed normally (using SAS, SAS Institute Inc., Cary, NC, USA). $P = 0{\cdot}05$ was considered significant.

Results

Defining the human T_{regs} population

The T_{regs} were identified as $CD4^+CD25^{high}$ T cells by selecting those $CD4^+$ cells whose CD25 expression exceeded the level of CD25 positivity seen on the CD4 negative population [31] (Fig. 1a). To characterize further the $CD4^+CD25^{high}$ T cells, different levels of expressions of the memory marker CD45RO, inhibitory receptor CTLA-4, death receptor CD95, early activation marker CD69 and homing receptor CD62L were compared among the $CD4^+CD25^-$, $CD4^+CD25^{int}$ and $CD4^+CD25^{high}$ subsets (Fig. 1b). The highest percentage of $CD45RO^+$, CTLA-4^+, $CD95^+$ or $CD62L^+$ cells was detected in the $CD4^+CD25^{high}$ subsets, and the percentages were 91% ± 2·4% (range: 89–95%), 87% ± 3·4% (range: 84–92%), 82% ± 4·1% (range: 79–86%) and 84% ± 3·1% (range: 81–88%) respectively. CD69 was not expressed. In agreement with previous reports, the expression levels of all of these markers were unchanged on the $CD4^+CD25^{high}$ T cells from either healthy controls or patients with HFRS, thereby allowing identification of these cells as T_{regs}. The terms $CD4^+CD25^{high}$ T cells and T_{regs} will be used synonymously throughout the study.

mRNA expression of FoxP3 in T_{regs} from HFRS patients

In our study, $CD4^+CD25^{high}$ T cells were highly purified using a modified protocol (described in Materials and methods). Optimization of this bead-based method allowed us to isolate $CD4^+CD25^{high}$ T cells rapidly and the purity of the separated $CD4^+CD25^{high}$ and $CD4^+CD25^-$ cell subsets was confirmed to be > 90% by flow cytometry. Previous studies suggested that FoxP3 is expressed selectively in T_{regs}, so we also used real-time revese transcription (RT)–PCR to quantify the expression levels of FoxP3 to confirm further that the purity of $CD4^+CD25^{high}$ cells isolated magnetically from both HFRS and healthy individuals are uniform. We found that $CD4^+CD25^{high}$ T cells isolated from acute-stage HFRS patients (HFRS1, $n = 20$), convalescent-phase patients (HFRS2, $n = 20$) and healthy individuals (control, $n = 20$) exhibited equal FoxP3 expression levels (Fig. 1c). Thus, we confirmed that the gated $CD4^+CD25^{high}$ T cells are indeed T_{regs} and not activated $CD25^+$ T cells.

Circulating T_{regs} numbers are reduced in acute-stage HFRS patients

The proportions and numbers of $CD4^+CD25^{high}$ T cells in HFRS1 ($n = 76$), HFRS2 ($n = 65$) and healthy controls ($n = 30$) are shown in Table 2 and Fig. 2. The percentage of $CD4^+CD25^{high}$ T cells in the $CD4^+$ population was reduced in HFRS1 [1·7% ± 0·4% (range: 1·4–3·5%)] compared with healthy controls [2·9% ± 0·4% (range: 1·8–4·3%)]. The total number of circulating $CD4^+CD25^{high}$ T cells was also lower

Table 2. Circulating cell populations in patients with haemorrhagic fever with renal syndrome (HFRS) at initial sampling (HFRS 1), follow-up (HFRS 2) and healthy controls.

Surface marker	HFRS1	HFRS2	Control
$CD4^+CD25^{high}$ T (%$CD4^+$)	1·7% ± 0·4%*,**	2·8% ± 0·6%	2·9% ± 0·4%
$CD4^+CD25^+$ T (%$CD4^+$)	28·7% ± 7·4%*,**	21·6% ± 3·5%	20·5% ± 3·9%
Number of $CD4^+CD25^{high}$ T (cells/μl)	26·1 ± 3·8*,**	39·6 ± 6·3	39·9 ± 6·2
Number of $CD4^+CD25^+$ T (cells/μl)	435·9 ± 12·1*,**	301·5 ± 11·4	289·1 ± 12·1

*$P < 0·01$. *P*-values refer to comparisons between the patients with HFRS and controls. **$P < 0·01$. *P*-values refer to comparisons between HFRS1 and HFRS2.

in HFRS1 [26·1 ± 3·8 cells/μl (range: 20·1–34·5 cells/μl)] compared with healthy controls [39·9 ± 6·2 cells/μl (range: 24·7–46·2 cells/μl)]. The proportions and numbers of $CD4^+CD25^{high}$ T cells in the acute phase of illness, between 2 and 12 days (mean 10 days) after the onset of HFRS, were not different (Fig. 2a and b). Interestingly, there was a significant increase in the percentage and numbers of $CD4^+CD25^{high}$ T cells in HFRS2 [2·8% ± 0·6% (range: 1·9–4·5%), 39·6 ± 6·3 cells/μl (range: 22·1–48·4) respectively] (Fig. 2c and d). The percentage of $CD4^+CD25^+$ T cells in the $CD4^+$ population was higher in HFRS1 [28·7% ± 7·4% (range: 20·3–34·1%)] compared with HFRS2 [21·6% ± 3·5% (range: 19·1–24·2%)] and healthy controls [20·5% ± 3·9% (range: 18·7–24·6%)]. The total number of circulating $CD4^+CD25^+$ T cells was also higher in HFRS1 [435·9 ± 12·1 cells/μl (range: 401·5–512·3 cells/μl)] compared with HFRS2 [301·5 ± 11·4 (range: 253·1–361·4 cells/μl)] and healthy controls [289·1 ± 12·1 cells/μl (range: 231·6–312·4 cells/μl)].

Decreased frequency of $CD4^+CD25^+FoxP3^+$ T cells in HFRS patients

To determine further the change in the number of T_{regs}, we used intracellular staining for FoxP3 protein and to estimate the percentage of $CD4^+CD25^+FoxP3^+$ T cells in $CD4^+$ and $CD4^+CD25^+$ T cells from HFRS1 ($n = 76$), HFRS2 ($n = 65$) and healthy controls ($n = 30$) using flow cytometry. Figure 3 (a,b) shows representative fluorescence activated cell sorter (FACS) pictures from a single HFRS patient and healthy control. As expected, during acute stage the percentage of $CD4^+CD25^+FoxP3^+$ T cells in $CD4^+$ T cells [1·6% ± 0·4% (range: 1·4–2·1%)] or $CD4^+CD25^+$ T cells [16·5% ± 4·2% (range: 12·1–19·2%)] from patients with HFRS was decreased significantly compared with healthy controls [2·3% ± 0·4%, 24·7% ± 4·7% respectively (range: 2·1–2·7%, 20·3–27·6% respectively)]. After treatment, the percentage of $CD4^+CD25^+FoxP3^+$ T cells in $CD4^+$ T cells

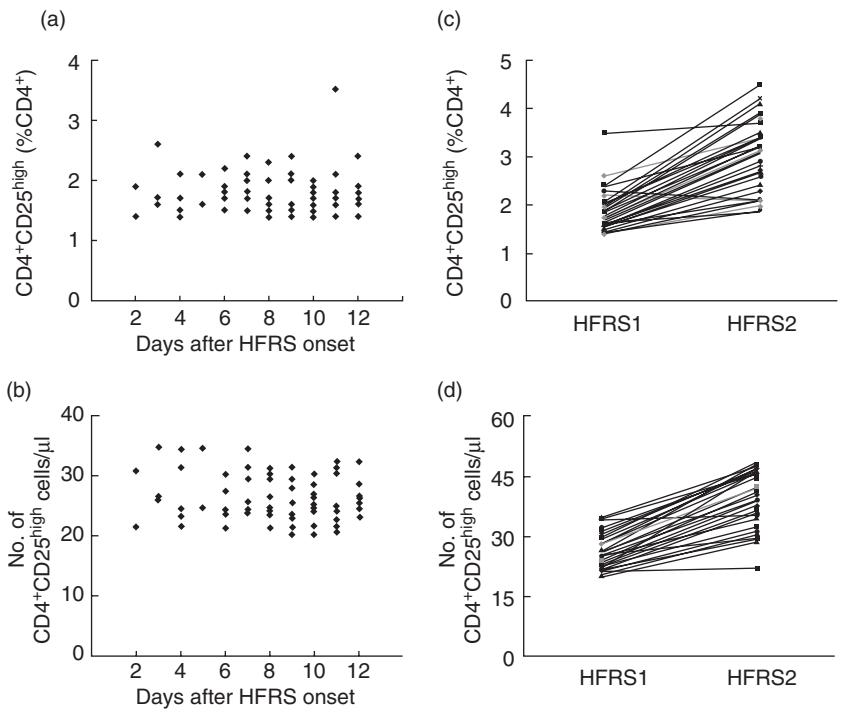

Fig. 2. The proportions and numbers of $CD4^+CD25^{high}$ T cells in haemorrhagic fever with renal syndrome (HFRS). (a,b) The proportions and numbers of $CD4^+CD25^{high}$ T cells in the acute phase of illness between days 2 and 12 (mean 10 days) after the onset of HFRS were not different. (c,d) The proportions and numbers of $CD4^+CD25^{high}$ T cells were increased significantly in HFRS2.

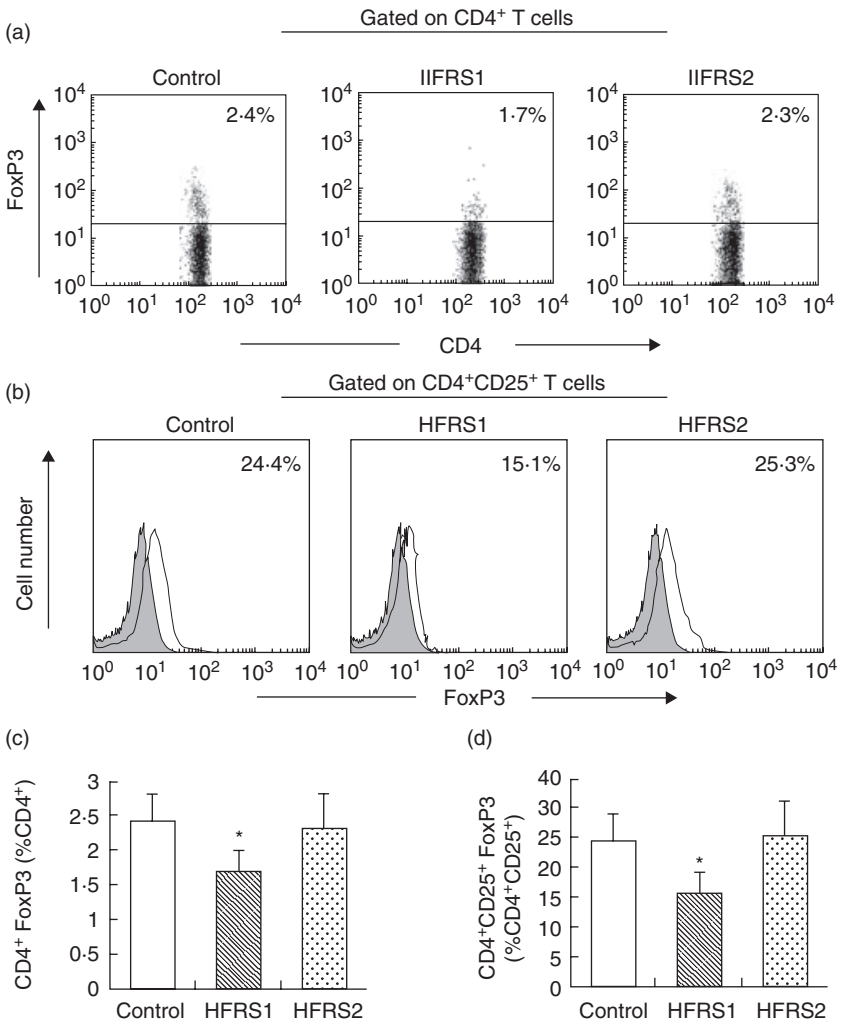

Fig. 3. The percentage of $CD4^+$ forkhead box P3 ($FoxP3^+$) T cells in $CD4^+$ and $CD4^+CD25^+FoxP3^+$ T cells in $CD4^+CD25^+$ T cells from haemorrhagic fever with renal syndrome 1 (HFRS1) ($n = 76$) was decreased significantly compared with healthy controls ($n = 30$), but there was a significant increase in HFRS2 ($n = 65$). $^*P < 0{\cdot}01$. (a,b) Representative fluorescence activated cell sorter pictures from a single HFRS patient and a healthy donor. Filled histograms represent staining with secondary antibody alone. (c) Averaged percentage of $CD4^+FoxP3^+$ cells in $CD4^+$ T cell subsets from HFRS1, HFRS2 and healthy controls by intracellular staining for FoxP3 protein. (d) Averaged percentage of $CD4^+CD25^+FoxP3^+$ cells in $CD4^+CD25^+$ T cell subsets from HFRS1, HFRS2 and healthy controls by standard deviation; $^*P < 0{\cdot}01$.

[2·2% ± 0·7% (range: 1·9–2·8%)] and $CD4^+CD25^+$ T cells [25·4% ± 4·7% (range: 20·1–29·9%)] were elevated significantly in convalescent phase patients (Fig. 3c and d). This showed that only a subset of the $CD4^+CD25^+$ T cell population may be $CD4^+$ T_{regs}.

Correlation of laboratory values with frequency of T_{regs}

The mean platelet count, BUN and serum creatinine of patients with HFRS are shown in Table 1. The frequency of T_{regs} was correlated positively with platelet count ($r = 0{\cdot}515$, $P < 0{\cdot}001$) and correlated negatively with BUN ($r = -0{\cdot}472$, $P < 0{\cdot}001$), serum creatinine ($r = -0{\cdot}379$, $P < 0{\cdot}001$) and AST ($r = -0{\cdot}263$, $P = 0{\cdot}002$).

Regulatory function of T_{regs} from HFRS patients

Quantitative analysis of the regulatory function of $CD4^+CD25^{high}$ T cells was performed by co-culture with autologous T responder cells at different ratios. The assay was repeated for all subjects. $CD4^+CD25^-$ (responder) cells from HFRS1, HFRS2 and healthy controls exhibited a similar strong proliferation to soluble anti-CD3 stimulus, while $CD4^+CD25^{high}$ (suppressor) cells were anergic to this stimulation (data not shown). When $CD4^+CD25^{high}$ regulatory T cells were co-cultured with autologous responder cells at different ratios (responder : suppressor ratios: 0:1, 1:1, 1:1/2, 1:1/4, 1:1/8 and 1:0), a dose-dependent suppression of proliferation was observed in both patients and healthy controls (Fig. 4a). In HFRS1, HFRS2 and healthy controls, $CD4^+CD25^{high}$ regulatory T cells suppressed $CD4^+CD25^-$ responder T cell proliferation consistently. Increasing the ratio of responder : suppressor T cells resulted in less suppression. No significant differences were detected between patients and healthy controls under the conditions we tested (Fig. 4a).

The T_{regs} suppress the production of cytokine

Besides potently suppressing the proliferation of effector $CD4^+CD25^-$ T cells, T_{regs} can also suppress the production of proinflammatory cytokines. Therefore, it is important to

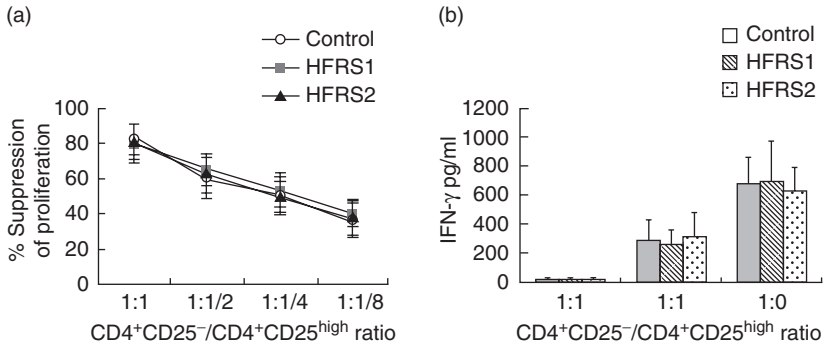

Fig. 4. T regulatory cells (T_{regs}) from patients with haemorrhagic fever with renal syndrome (HFRS) maintain their regulatory function. (a) $CD4^+CD25^{high}$ T cells from HFRS1 ($n = 15$), HFRS2 ($n = 15$) and healthy controls ($n = 15$) exhibited equal suppressor activity at different ratios of responder/suppressor T cells. (b) Supernatants from $CD4^+CD25^{high}$ T cells or $CD4^+CD25^-$ T cells cultured alone or from co-culture of both populations at a 1 : 1 ratio were evaluated at day 4. Data represent 20 different experiments. $^*P < 0{\cdot}01$.

determine whether there is any defect in suppressing cytokine productions from T helper cells by T_{regs} from HFRS patients. We analysed the concentrations of cytokines in the supernatants obtained from the co-culture of $CD4^+CD25^{high}$ T cells and $CD4^+CD25^-$ T cells. As shown in Fig. 4b, $CD4^+CD25^-$ T cells cultured alone produced large amounts of IFN-γ from HFRS1, HFRS2 and healthy controls. Supernatants from cultures of $CD4^+CD25^{high}$ T cells alone with antigenpresenting cells contained few IFN-γ. Co-culture of $CD4^+CD25^{high}$ T cells with $CD4^+CD25^-$ T cells at a 1:1 ratio resulted in significant inhibition of IFN-γ secretion in the culture supernatants from healthy controls and patients with HFRS. This suggests that $CD4^+CD25^{high}$ T cells from HFRS1, HFRS2 and healthy controls are effective in suppressing cytokine production IFN-γ in a similar degree.

Discussion

The T_{regs} may influence the immune response to infectious pathogens [32,33]. The relationship between T_{regs} and infections is complex and remain poorly understood, although the role of T_{regs} responses in controlling tissue damaging inflammatory reactions has been described in several human infections as well as in numerous model infection systems in mice. So far, little is known about the properties of T_{regs} in patients with HFRS.

Our data demonstrate that the number of T_{regs} in acute-stage HFRS patients is decreased significantly. When patients' condition stabilized following therapy, the number of T_{regs} in the patients' peripheral blood returned to normal value by the time we obtained the second blood sample. Our finding is different from that of the only other study to investigate $CD4^+CD25^+$ cells in HFRS. Huang *et al.* found that the ratio of $CD4^+CD25^+$ lymphocytes in the acute phase of HFRS was higher than that in convalescent phase [26]. The explanation for the discrepancy is that in their study $CD4^+CD25^+$ cells contained $CD4^+CD25^{int}$ cells and $CD4^+CD25^{high}$ cells and they did not distinguish T_{regs} from activated $CD4^+CD25^+$ cells. In our study, the proportions and numbers of $CD4^+CD25^+$ cells, which contained $CD4^+CD25^{int}$ cells and $CD4^+CD25^{high}$ cells, was also higher in HFRS1. CD25 is a marker for T_{regs} in humans, but this molecule also represents an activation marker of effector T cells. Studies have shown that in human peripheral blood, regulatory activity is associated with the small fraction of $CD4^+$ T cells expressing the CD25 bright phenotype and most of them were CTLA4-positive and expressed CD45RO, CD95 or CD62L and CD69 was not expressed [6,31]. The best marker for distinction is FoxP3, because it can distinguish T_{regs} from T cells without regulatory function that are also present in the $CD4^+CD25^+$ T cell pool. To control our CD25-based gating, we used real-time RT–PCR to quantify the levels of FoxP3 within $CD4^+CD25^{high}$ T cells. All patients had a typical $CD4^+CD25^{high}$ T cell population, displaying similar phenotype. Most importantly, we found no difference on the FoxP3 mRNA expression levels of this population between controls and patients, thereby allowing identification of these cells as T_{regs}.

Functional analysis demonstrated that T_{regs} from patients with HFRS maintain their regulatory function. In our experiments, they can suppress the proliferation and cytokine secretion of $CD4^+$ effector T cells in a manner equally efficient to those from healthy controls. Proliferation was reduced drastically upon the addition of T_{reg} cells. Co-culture is currently accepted methods for assessment T_{regs} function *in vitro* [8]. In agreement with our data, Putnam *et al.* [34] also reported that stimulation with soluble anti-CD3 plus soluble anti-CD28 or low doses of plate-bound anti-CD3 resulted in similar suppression of $CD4^+CD25^{high}$ T cells and $CD4^+CD25^-$ T responder cells co-cultures in type 1 diabetic and normal subjects. Huan *et al.* found that FoxP3 message and protein expression levels in peripheral T_{regs} were related quantitatively to a reduction in functional suppression of T_{regs} [35]. Therefore, the intact regulatory function of T_{regs} in HFRS patients may be related to normal FoxP3 message expression levels. Based on these results, we conclude that T_{regs} are fully functional in acute HFRS. We suggest that the immunopathology in HFRS is not caused by defective T_{reg} function.

The balance between regulatory and effector functions determines the outcome of disease. In general, regulatory T_{reg} responses benefit the host by reducing the collateral tissue damage that usually accompanies immune responses to infections and which in some circumstances are fully responsible for any lesions observed. Under some circumstances the T_{reg} response is also considered as detrimental to the host,

because a prompt and strong T_{reg} response may impair protective immune response to infectious agents. The outcome of such immune blunting may be that the agent is more able to persist which, in some infections, such as hepatitis C and hepatitis B infections in humans [36–38], can result in chronic tissue damage. Studies showed that HFRS is due to immunologically mediated capillary leakage in target organs. Increased numbers of $CD8^+$ cells and the release of proinflammatory cytokines have been implicated in the HFRS disease process [24,39]. Terajima *et al.* hypothesize that the protection of endothelial cells may be overwhelmed by excess amounts of activated $CD8^+$ T cells, which may be the mechanism of capillary leakage in HPS and HFRS patients [40]. A reduced T_{reg} frequency may result in insufficient suppression of the effector functions of activated T cells and failure to terminate the immune responses, which predispose to the development of immunopathology in HFRS. In contrast, increased numbers of T_{regs} may play an important role in limiting immunopathology in the natural rodent reservoirs of Hantaviruses, which make infected hosts experience few or no pathological changes, and the host rodent can remain infected persistently for life [41,42]. The mechanisms causing haemorrhage are likely to vary among viruses. Lühn found T_{reg} frequencies and also T_{reg} : effector T cell ratios were increased in patients with acute infection and a relative rise of T_{reg} : effector T cell ratios is beneficial for disease [43]. The value of T_{reg} responses in controlling tissue damaging inflammatory reactions has also been described in other infections. In the infections of cornea of the eye with HSV, the chronic inflammatory reaction appears to be caused by an immunopathological response to the pathogen. Suvas *et al.* [44] have shown that the severity of the lesion is influenced beneficially by FoxP3 T_{regs} because animals depleted of such cells develop more severe disease and are susceptible to lower doses of infection. In the current study, we failed to explain why T_{regs} are decreased in the peripheral blood of acute-stage HFRS patients. A possibility is that T_{reg} generation is decreased. T_{regs} are produced mainly in the thymus but can also be induced in the periphery under adequate conditions. In HFRS patients multiple factors include Hantaviruses; other cells or cytokines may impair their immediate environment for their differentiation and lead possibly to suppress their generation transiently in the periphery. Another possibility is that circulating T_{regs} may be recruited rapidly to infected organs and/or into lymph nodes, but current evidence suggests that the major site of disease pathogenesis is in the blood. Therefore, further studies are needed.

The present data imply that reduced circulating T_{regs} may be associated with the pathogenesis of HFRS. Reversible numbers and intact function of T_{regs} in the peripheral blood of HFRS patients contribute presumably to the disease course. Manipulating strategically the T_{reg} responses to achieve a favourable balanced relationship between the host and the infecting agent may prove to have great clinical benefit.

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