

Both tacrolimus and sirolimus decrease Th1/Th2 ratio, and increase regulatory T lymphocytes in the liver after ischemia/reperfusion

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The protective effects of immunosuppressants against ischemia/reperfusion (I/R) injury have been attributed to their non-specific anti-inflammatory effect. However, these effects may also depend on their effect on T lymphocytes, which are increasingly considered to be key players in I/R. Here, we studied the effects of tacrolimus and sirolimus on lymphocyte subpopulations in an I/R rat model. The animals were treated with tacrolimus, sirolimus or vehicle, before undergoing a 60-min ischemia event of the right hepatic lobe, followed by excision of the remaining liver. After 2 h, I/R rats showed increased mortality, plasma lactate dehydrogenase (LDH) levels, hepatocyte apoptosis, liver histological injury and parenchymal infiltration by neutrophils, macrophages, NK cells and T lymphocytes. Most of the changes were antagonized by both immunosuppressants. Tacrolimus augmented the proportion of cycling cells in I/R rats, whereas sirolimus showed the opposite effect. The increased Th1/Th2 ratio found in I/R livers after 2 h was reverted by immunosuppressants, which also amplified the proportion of CD4⁺CD25⁺Foxp3⁺ regulatory T lymphocytes at 24 h.

The protective effects of both tacrolimus and sirolimus correlated well with a decreased ratio of proinflammatory to anti-inflammatory T lymphocytes, and with an increase in the Treg proportion. This suggests a new mechanism to explain the known beneficial effect shown by immunosuppressants early after I/R.

Laboratory Investigation (2009) 89, 433–445; doi:10.1038/labinvest.2009.3; published online 2 February 2009

KEYWORDS: ischemia/reperfusion; liver regeneration; regulatory T lymphocytes; sirolimus; tacrolimus; Th1/Th2 ratio

Major liver tissue loss associated with periods of ischemia is a common feature in trauma, hepatic surgery and transplantation. The actions of polymorphonuclear leukocytes (PMN) on liver ischemia/reperfusion (I/R) phenomena have been well studied. PMN are considered to be the primary cells responsible for propagating the injury response.¹

There is increasing evidence that T lymphocytes, in addition to their well-known role in transplant rejection, are also key players in the acute I/R injury of the graft.^{2,3} In fact, they can secrete several cytokines, including TNF α , IFN γ , IL-4 and GM-CSF,^{4,5} which could participate in PMN-dependent inflammatory responses. Also, nude, CD4⁺-depleted, or CD4⁺- or CD8⁺-knockout mice suffer a less intense I/R injury in their liver or kidneys than the immunocompetent controls.^{2,3} The growing role attributed to lymphocytes in the I/R phenomena would be of particular relevance in the liver, as hepatic parenchyma is especially rich on T lymphocytes

and NK cells, which are also known to be carried from the donor to the recipient during liver transplantation.⁶

Tacrolimus, a widely used immunosuppressant, blocks IL-2 transcription by inhibiting calcineurin. Like most calcineurin inhibitors, besides inhibiting T lymphocytes, it possesses an unspecific anti-inflammatory effect, partly through its action on other defensive cells such as monocytes and PMN.⁷ This effect has been used to explain the observed beneficial effects of these drugs on liver I/R models.^{1,8} However, tacrolimus could also modify the early allocation of T-lymphocyte subpopulations into the liver parenchyma, and this would be an additional mechanism to modulate acute liver inflammation. Thus, we designed this study to determine whether the effect of tacrolimus on an I/R plus hepatectomy model is accompanied by changes in the lymphocyte subpopulations of the liver. Sirolimus does not inhibit calcineurin, but a cell cycle regulatory protein, the

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Received 15 September 2008; revised 1 December 2008; accepted 2 December 2008

mammalian target of rapamycin (mTOR). We also studied whether this specific signaling pathway results in a different effect in our model.

The results of this work show that both tacrolimus and sirolimus have a beneficial effect in liver I/R injury. This effect is accompanied by a short-term decrease in the ratio of proinflammatory to anti-inflammatory T lymphocytes, and by a progressive increase in the proportion of regulatory T lymphocytes, suggesting a common mechanism by which different immunosuppressive drugs could protect the liver early after I/R injury.

MATERIALS AND METHODS

Animals

Sprague–Dawley male rats, weighing 250–300 g, housed and fed under standard laboratory conditions, were used. All animals received humane care, with free access to food and water. They were pretreated with tacrolimus (6 mg/kg, Calbiochem, Darmstadt, Germany) (T), sirolimus (2 mg/kg, Calbiochem) (S) or vehicle (5% propylene glycol and 2% Tween-80 in saline). After 60 min, they underwent a 60-min ischemia event of the right lateral lobe by means of a microvascular clamp. On restoration of flow, the median and left lateral lobes (about 80% of the total liver mass) were excised.⁹ Other animals underwent hepatectomy alone or a sham operation (Table 1). Enoxaparin (30 mg/kg) was administered subcutaneously 60 min before surgery in all rats.

Animals were killed 2 or 24 h later, and plasma, liver and submaxillary lymph nodes (LN) were obtained. Plasma lactate dehydrogenase (LDH) was determined spectrophotometrically by using a specific kit (Sigma, Spain).

Isolation of Hepatic Lymphoid Cells

Livers were minced into small fragments and washed free of blood in cold PBS. Fragments were passed through a 230- μ m steel mesh, and the resulting cell suspension was cotton-

filtered to remove tissue debris and resuspended in RPMI-1640 plus 5% FCS. Leukocytes were isolated by density-gradient centrifugation (Histopaque-1077, Sigma). Cell viability was assessed by trypan blue staining.

Flow Cytometric Analysis

Mouse anti-rat mAbs of the following specificities, labeled with FITC, PE, PE-Cy5 or PerCP, were obtained from BD PharMingen (San Diego, CA, USA) and Serotec (Oxford, UK): CD4, CD5, CD8 α , CD8 β , CD25, CD45RC, NKR-P1A, OX12, TCR $\alpha\beta$ and TCR $\gamma\delta$. Three-color flow cytometric analyses were performed using a FACScalibur (BD Biosciences, San Jose, CA, USA), as described earlier.¹⁰ For Foxp3-APC staining, a prior cell membrane permeabilization step was performed following the manufacturer's instructions (Cell Fixation/Permeabilization Kit, eBioscience, San Diego, CA, USA).

Histology, Immunofluorescence and Quantification of Apoptosis

Liver cryosections (5–7 μ m) were fixed in acetone (immunofluorescence) or paraformaldehyde (Ki67 or TUNEL). After blockade of the unspecific binding with donkey serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA), sections were incubated with His48, anti-CD5, anti-NKR-P1A (PharMingen), ED1, ED2 (European Collection of Animal Cell Cultures) or rabbit anti-laminin (Sigma) antibodies. Liver regeneration was determined using MIB-5 (anti-Ki67) (Dako, Barcelona, Spain), after heat-induced epitope retrieval in citrate buffer. Donkey FITC-conjugated anti-mouse or Texas Red-conjugated anti-rabbit (Jackson Laboratories, West Grove, PA, USA) served as secondary antibodies. Apoptosis was quantified by TUNEL (In situ Cell Death Detection Kit, Roche, Mannheim, Germany). Sections were mounted on Prolong Gold[®] (Invitrogen, Barcelona, Spain)

Table 1 Experimental groups

| | Sham | Hepatectomy | Ischemia/reperfusion |
|------------|--|---|---|
| Control | <i>Sham:</i> Vehicle 0.1 ml i.m. and 0.5 ml i.p. | <i>Hepatectomy:</i> Vehicle 0.1 ml i.m. and 0.5 ml i.p. | <i>I/R:</i> Vehicle 0.1 ml i.m. and 0.5 ml i.p. |
| Tacrolimus | <i>Sham+T:</i> Tacrolimus 6 mg/kg i.m. Vehicle 0.5 ml i.p. | <i>Hepatectomy+T:</i> Tacrolimus 6 mg/kg i.m. Vehicle 0.5 ml i.p. | <i>I/R+T:</i> Tacrolimus 6 mg/kg i.m. Vehicle 0.5 ml i.p. |
| Sirolimus | <i>Sham+S:</i> Vehicle 0.1 ml i.m. Sirolimus 2 mg/kg i.p. | <i>Hepatectomy+S:</i> Vehicle 0.1 ml i.m. Sirolimus 2 mg/kg i.p. | <i>I/R+S:</i> Vehicle 0.1 ml i.m. Sirolimus 2 mg/kg i.p. |

Abbreviations: i.m., intramuscular; i.p., intraperitoneal; I/R, ischemia/reperfusion; +T, plus tacrolimus treatment; +S, plus sirolimus treatment.

and examined under a fluorescence microscope, Zeiss Axioplan 2[®] (Oberkochen, Germany).

Liver sections stained with hematoxylin–eosin were scored using the system devised by Suzuki *et al.*¹ grading the tissue damage from 0 to 4 on the basis of the presence and severity of congestion, cytoplasmic vacuolization and cell necrosis.

For quantitative determinations, 10 non-overlapping fields were analyzed in a blinded manner for each of a minimum of six animals per group. They were randomly selected from four liver sections at least 1500 μm apart. A proportion of studied cells were quantified by the ImageJ[®] software (NIH, Bethesda, MD, USA), determining the positive cells/total nuclei (Hoechst-33342[®], Molecular Probes, Eugene, OR, USA) ratio in each field.

Statistical Analysis

Results are expressed as means (\pm s.e.m.) of at least six independent experiments. Comparison of means was performed by ANOVA (Tukey as *post-hoc* test), and survival analysis by the log-rank method (Statgraphics Centurion[®] software).

RESULTS

Both Tacrolimus and Sirolimus Increase Survival and Decrease Hepatocellular Injury Among I/R Rats

Initially we studied rat survival and hepatocellular injury, the latter through the analysis of plasma LDH levels, histological damage and apoptosis rate. Eight out of 20 animals in the I/R group died, whereas there was no mortality in the sham or hepatectomy groups. Tacrolimus or sirolimus treatment decreased both mortality and plasma LDH in the I/R group (Figure 1a and b).

Rats killed 2 or 24 h after I/R showed severe cytoplasmic vacuolization, sinusoidal congestion and parenchymal necrosis (Figure 1c and d), whereas milder changes were appreciable in the immunosuppressant pretreated rats. When quantified using the Suzuki's score,¹ the damage amelioration showed statistical significance already at 2 h (Figure 1e). The hepatocyte apoptosis rate detected by TUNEL was also higher after I/R (64.0 ± 4.8 per 1000) than after sham operation (0.73 ± 0.11 , $P < 0.01$) or hepatectomy (7.5 ± 0.8 , $P < 0.01$). It was again markedly decreased by tacrolimus or sirolimus treatment (33.4 ± 3.7 and 26.8 ± 1.8 , respectively, both $P < 0.01$ vs I/R control) (Figure 1f–h).

Collectively, these data show an early beneficial effect of both immunosuppressants in decreasing hepatocellular damage and rat mortality.

Both Drugs Display Opposite Effects on Liver Regeneration

The liver's capability to restore the hepatic functional mass could influence the consequences of I/R injury. Thus, a liver regeneration index and the effects of tacrolimus and sirolimus on this parameter were analyzed in our model. Hepatocyte proliferation was increased in all hepatectomy

and I/R rats as early as 2 h after the procedure. At 24 h, tacrolimus increased further the rate of cycling cells, whereas sirolimus decreased it (Figure 2).

Treated I/R Rats Show Less Infiltration by Inflammatory Cells

The I/R phenomena and the ensuing hepatocellular damage activated an inflammatory response characterized by a massive leukocyte infiltration, consisting mainly of PMN and monocytes, and also of lymphocytes and NK cells.

Liver neutrophils (HIS48⁺ cells) increased clearly at 2 h after I/R, both tacrolimus and sirolimus being very effective in attenuating PMN infiltration (Figure 3). At 24 h, the infiltrating PMN were mainly confined to the areas of necrosis and intergroup differences were difficult to quantify.

Although the number of macrophage/Kupffer (ED2⁺) cells did not differ between groups, that of monocytes/immature macrophages (ED1⁺) increased after I/R (30.4 ± 3.6 , I/R, vs 17.7 ± 2.8 , sham, 2 h, and 27.4 ± 3.3 , I/R, vs 15.6 ± 2.5 , sham, 24 h, per 1000, $P < 0.05$ for both). Immunosuppressants showed no effect on these cell populations.

Both hepatectomy and I/R rats showed an increased NK cell number (18.5 ± 2.1 , I/R, and 10.6 ± 1.0 , hepatectomy, vs 3.2 ± 0.6 , sham, per 1000, $P < 0.01$ for both), mainly located in and around the portal areas (Figure 4a). Such increase was partially reversed in pretreated I/R rats, being significant for sirolimus (to 7.5 ± 0.9 , $P < 0.05$) (Figure 4b and c). Intrahepatic NK cells returned to baseline at 24 h in all groups.

I/R induced an increase in parenchymal T cells at 2 h (15.8 ± 2.5 , I/R, vs 4.6 ± 0.4 , hepatectomy, and 2.9 ± 0.4 , sham, per 1000, $P < 0.01$ for both) and 24 h (5.9 ± 1.3 , I/R, vs 2.8 ± 0.4 , hepatectomy, and 2.0 ± 0.3 , sham, per 1000, $P < 0.05$ for both). This increase was attenuated by both immunosuppressants (5.6 ± 0.7 , I/R + T, and 9.5 ± 0.8 , I/R + S, per 1000, 2 h, $P < 0.05$ for both).

Therefore, tacrolimus or sirolimus can attenuate the increase in hepatic tissue infiltration by PMN, NK and T cells.

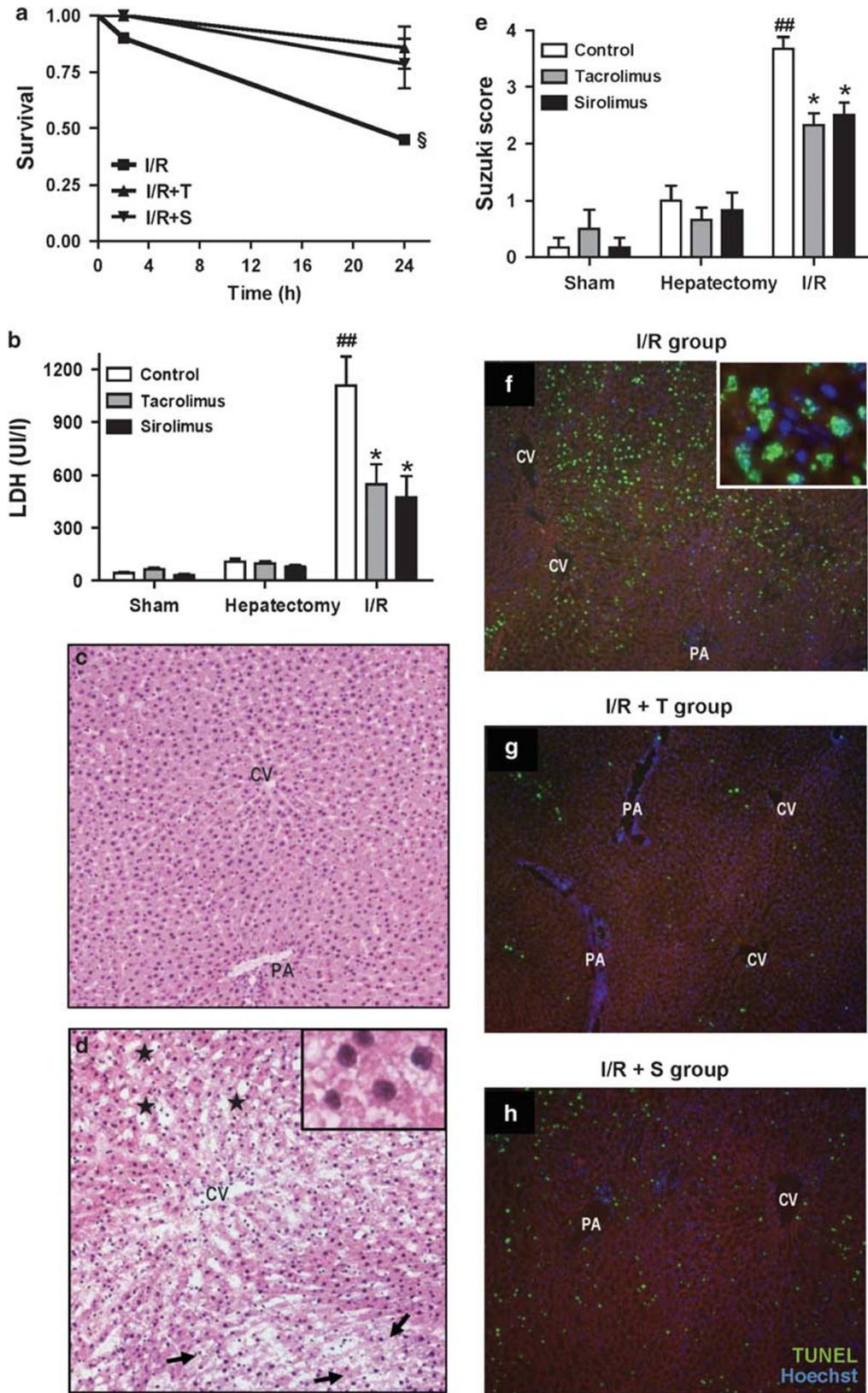
Immunosuppressants Modulate I/R-Induced Changes in Several Lymphocyte Subpopulations

A cytofluorometric analysis of the hepatic lymphocytes was performed to complement the immunofluorescence findings. In addition, we studied the submaxillary LN subpopulations to help distinguish a local from a systemic response.

Regarding NK and NKT cells, their proportions were higher in the liver than in the LN (Figure 5a), although without intergroup differences.

The proportion of total B lymphocytes was not influenced by hepatectomy or I/R, although it was decreased by both drugs at 2 h (Figure 5b). However, the B1 (OX12⁺CD5⁺) subpopulation increased at 2 and 24 h after I/R (Figure 5b), being antagonized by tacrolimus or sirolimus pretreatment.

The main subpopulations of T lymphocytes, including T $\alpha\beta$ CD4 and CD8 cells, as well as T $\gamma\delta$ cells, were also analyzed. Liver T $\gamma\delta$ cells were not modified by hepatectomy



or I/R, although both drugs decreased their proportion at 24 h. The effect of sirolimus was already evident at 2 h (Figure 5b).

The hepatic proportion of $\text{TCR}\alpha\beta^+$ cells experienced an early increase after I/R in all groups, with a parallel decrease in the LN, whereas no changes were detected at 24 h (Figure 6). At 2 or 24 h, we found no intergroup differences in the proportions of CD4 or CD8 cells (Figure 6).

We also analyzed liver and LN lymphocytes for other less prevalent T-cell subpopulations: immature $\text{CD4}^+\text{CD8}^+$ and

extrathymically differentiated $\text{CD8}\alpha\alpha$ lymphocytes, which are increased in several pathological situations. Neither hepatectomy nor I/R modified these subpopulations, which also remained unchanged in the drug pretreated groups (Figure 7).

Therefore, the subpopulations most affected by tacrolimus or sirolimus pretreatment are B, B1 and $\text{T}\gamma\delta$ cells.

Both Drugs Palliate the I/R-Induced Increase in Th1/Th2 Ratio

Depending on their pattern of secreted cytokines, two main classes of CD4 T lymphocytes are distinguished: Th1 and Th2, with pro- and anti-inflammatory properties, respectively. Both subpopulations differ in their CD45RC expression level.¹¹ Among hepatic $\text{TCR}\alpha\beta^+\text{CD4}^+$ lymphocytes, the ratio of proportion of Th1 ($\text{CD45RC}^{\text{high}}$) cells to that of Th2 ($\text{CD45RC}^{\text{low}}$) cells was increased 2 h after I/R, a finding that was antagonized by both drugs (Figure 8). However, these changes were not observed in animals killed at 24 h, neither were they mirrored in the submaxillary LN, suggesting a local, acute, short-term response.

Treg Cells Experienced a Late Increase in Treated Groups

Another T-lymphocyte subpopulation, the Treg, is being increasingly involved in modulating diverse inflammatory responses. Regulatory T lymphocytes are characterized by their expression of CD25 and the transcriptional repressor Foxp3.¹² Although we found no changes in the proportion of $\text{CD4}^+\text{CD25}^+\text{FoxP3}^+$ cells in the liver or LN at 2 h, tacrolimus and sirolimus increased the Treg percentage at 24 h after I/R, whereas sirolimus showed a similar effect also in hepatectomy and sham groups (Figure 9).

DISCUSSION

Reperfusion injury plays an important role in the development of primary nonfunctioning of the liver graft after transplantation, having an incidence ranging from 6 to 9.2%,^{13,14} and requiring liver retransplantation for the patient to survive. In this study, just 8 out of 20 untreated I/R rats died; thus, our model might resemble a less severe condition, but it permits enough sensitivity for assessing changes in the animal survival rate.

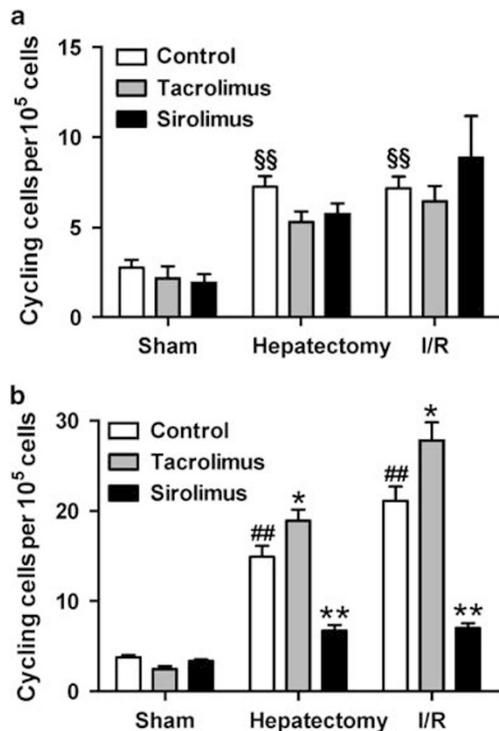


Figure 2 The hepatocyte proliferation rate experiences an opposite behavior after drug treatments. Ki-67^+ proliferating hepatocytes were determined at 2 (a) and 24 (b) hours after the interventions by immunofluorescence contrasted with Hoechst 33342 and laminin. The graphs represent the values obtained from different rats group showing that hepatectomy and ischemia/reperfusion (I/R) trigger an increase in hepatocyte proliferation, which is augmented by tacrolimus and reduced by sirolimus at 24 h. Each bar in the graphs represents the mean and s.e. of values found in at least six animals. $^*P < 0.05$ vs its control; $^{**}P < 0.01$ vs its control; $^{##}P < 0.01$ vs the other untreated controls; $^{$$}P < 0.01$ vs sham control.

Figure 1 Animal survival and hepatocellular injury are improved by tacrolimus and sirolimus. (a) Survival curves of rats from the different ischemia/reperfusion (I/R) groups ($N = 20$ rats/group), compared according to log-rank (Mantel-Cox) test. Tacrolimus and sirolimus increase the survival of rats undergoing I/R injury. (b) Both drugs antagonize the increase in plasma lactate dehydrogenase (LDH) levels showed by I/R rats killed at 2 h. (c, d) Hematoxylin-eosin-stained cryosections of I/R livers (d) showed signs of tissue damage, such as congestion (*), cytoplasmic vacuolization (inset) and necrosis (arrow), when compared with control livers (c). These lesions were quantified by Suzuki's score (e). There was a decrease in the tissue damage score at 2 h in treated I/R rats. (f-h) Liver samples from the different groups were collected for TUNEL and the nuclei were detected by Hoechst 33342 to quantify the proportion of apoptotic cells. The photographs show homogeneous distribution of the positive cells representative of liver cryosections from I/R (f), I/R + T (g), and I/R + S (h) groups. The inset photograph in (f) shows the restricted nuclear staining obtained for TUNEL. Both drugs markedly reduced the apoptosis rate found among the hepatocytes. $^{\$}P < 0.05$ vs the rest; $^*P < 0.05$ vs control; $^{##}P < 0.01$ vs the other untreated controls. (c, d) Original magnification $\times 200$ (inset, $\times 1000$). (f-h) Original magnification $\times 100$ (inset, $\times 1000$). Each data point in b and e graphs represents the mean and s.e. of values found in at least six animals. PA: portal area; CV: central vein; +T: plus tacrolimus treatment; +S: plus sirolimus treatment.

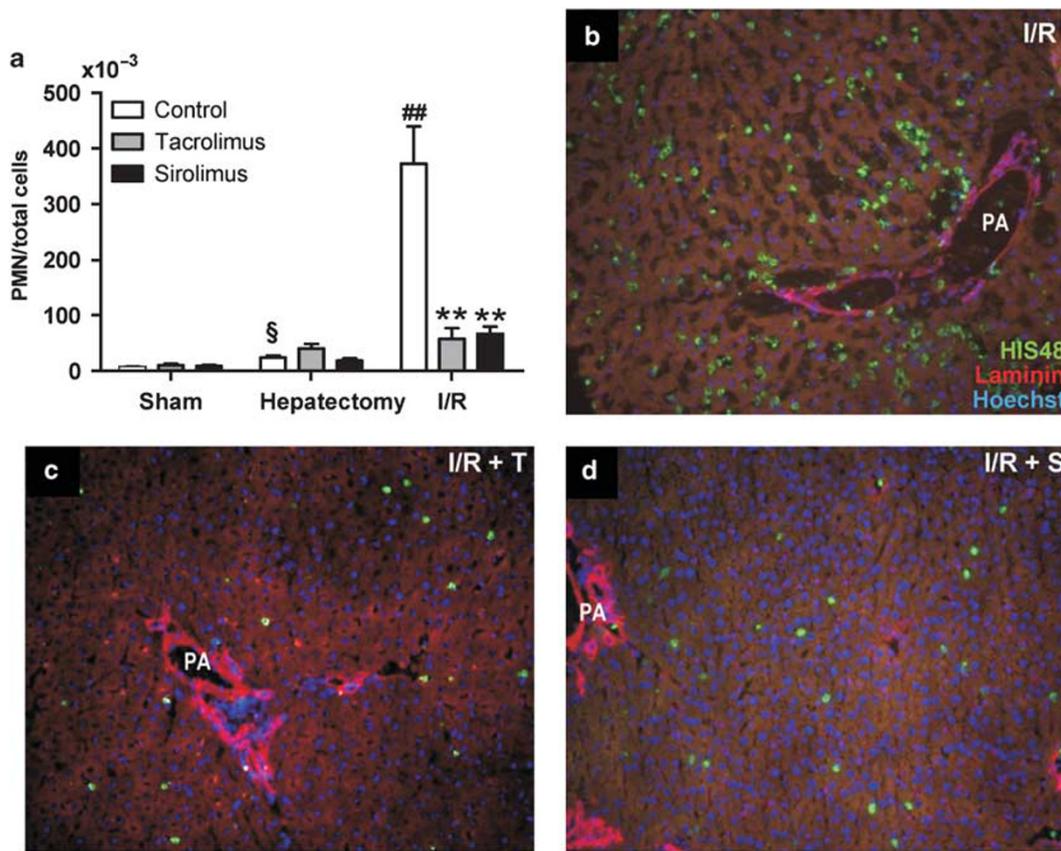


Figure 3 Decreased hepatic neutrophil recruitment in ischemia/reperfusion (I/R) after tacrolimus or sirolimus administration. Three-color immunofluorescence analyses (b–d) were carried out to determine the proportion (a) and distribution of polymorphonuclear leukocytes (PMN, HIS-48⁺ cells) in the liver parenchyma. Liver cryosections were marked with anti-laminin antibody and anti-HIS 48, and the nuclei were labeled with Hoechst 33342. The photographs (b–d) are representative of immunofluorescence staining obtained in livers from rats of the I/R (b), I/R + T (c) and I/R + S (d) groups killed 2 h after the intervention. The PMN appear homogeneously distributed through the parenchyma in all groups, but their number was drastically increased in the untreated I/R rats (b). Original magnification × 200. Each bar in graph represents the mean and s.e. of values found in at least six animals killed at 2 h. ***P* < 0.01 vs its control; ##*P* < 0.01 vs the other controls; §*P* < 0.05 vs sham control. PA: portal area.

T lymphocytes are increasingly considered to participate in the inflammatory injury secondary to I/R,^{2,15,16} although their implication is not completely out of question.¹⁷ Attachment of circulating lymphocytes to the hepatic sinusoids has been observed early after the reperfusion,¹⁸ which could explain why we found them increased in the liver parenchyma as early as 2 h. In addition, a human liver contains about 10¹⁰ lymphocytes, and passenger lymphocytes seem to play a key pathogenic role in the reperfusion that occurs after cold ischemia of the liver.¹⁶ Nevertheless, the role of these resident lymphocytes vs peripheral ones or the interrelation between both populations remains unknown.

The effect of T lymphocytes is probably multifactorial as suggested by the fact that most systemic immunosuppressants attenuate this injury.¹⁵ Although both CD4⁺ and CD8⁺ T lymphocytes seem to contribute to the microvascular damage after intestinal I/R,¹⁸ one key aspect would be the action of CD4⁺ lymphocytes as a determinant of the subacute PMN infiltration.² This PMN recruitment might depend on a direct action of CD4⁺ lymphocytes,

through their capacity to produce several cytokines,^{4,18} or in an indirect way, through the stimulus to produce chemotactic factors by Kupffer cells.¹⁵ However, a beneficial effect has also been observed with peripheral T-lymphocyte depletion, without concomitant modifications in hepatic PMN, during I/R injury.¹⁹ Thus, the pathogenic role of T lymphocytes does not seem to be limited to PMN recruitment, and may involve other lymphoid populations.

B lymphocytes have also been implicated in the I/R injury.²⁰ Although we found no changes in total B lymphocytes, the B1 subpopulation, which was found pathogenic after intestinal reperfusion,²¹ experienced an early increase after I/R. Plausibly, other lymphocyte subpopulations also related to innate immunity, including Tγδ and NKT cells, could play a role in I/R, which is considered to be an antigen-independent condition.²² Tγδ lymphocytes can survey, among other antigens, heat-shock proteins and modified cell components, and they seem to play an important role in the early stages of systemic inflammatory response syndrome and sepsis.²³ However, the higher level of aggression of our

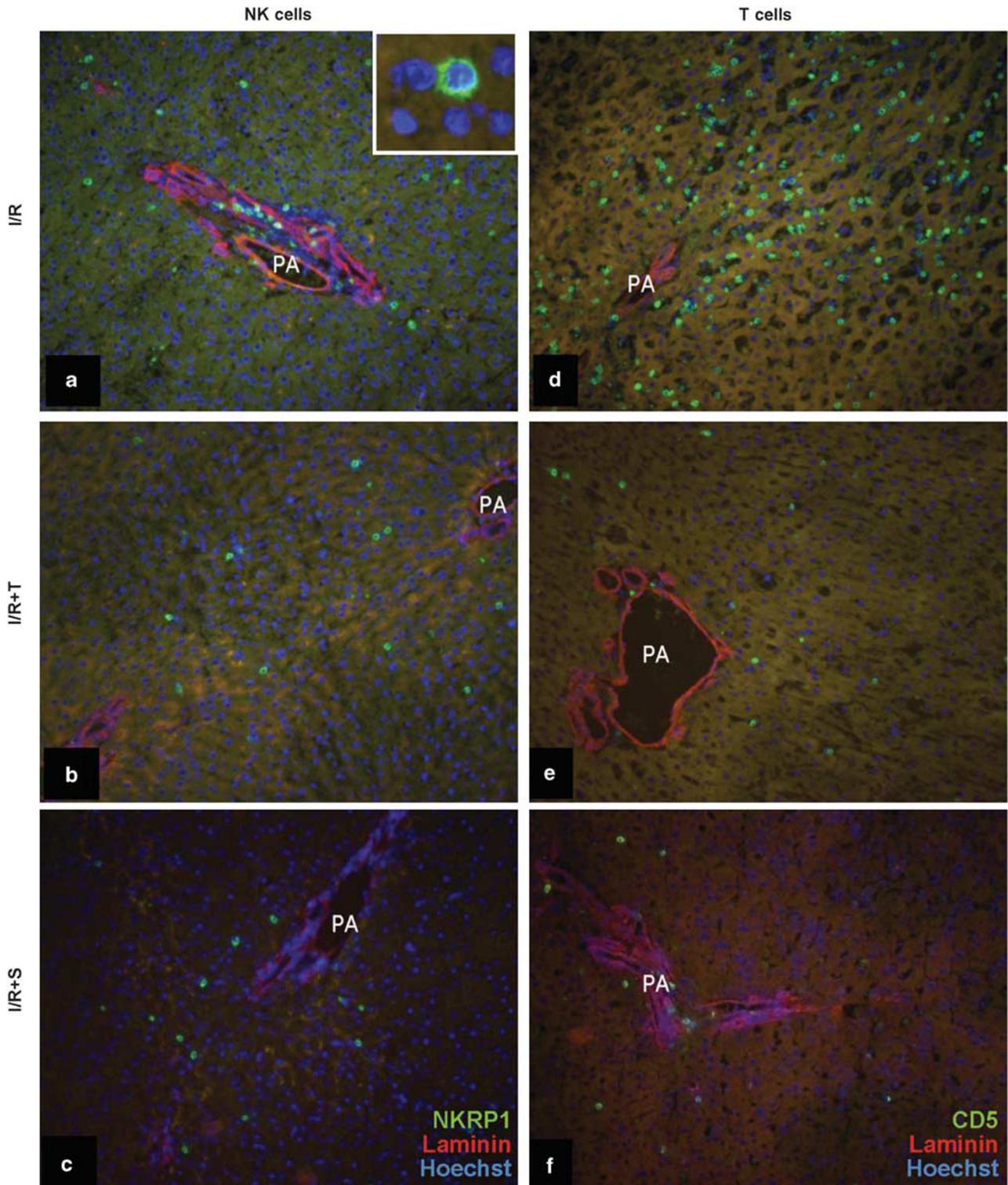


Figure 4 Both sirolimus and tacrolimus decreased ischemia/reperfusion (I/R)-induced hepatic infiltration by NK and T cells. Hepatic cryosections from different I/R groups, killed 2 h after reperfusion, were stained for laminin (expressed in the basal lamina of blood vessels), nuclei (Hoechst 33342), and NK cells (anti-NKR-P1A) (photographs **a–c**) or T cells (anti-CD5) (photographs **d, e**). A detail of a cell with NKR-P1A positive staining is shown in the inset in (**a**). Control I/R livers show a higher proportion of both NK and T cells, distributed mainly on the portal areas (PA) or in the parenchyma, respectively, than the groups treated with tacrolimus or sirolimus. The images are representative examples of at least six animals per group. Original magnifications (**a–f**) $\times 200$ and inset $\times 1000$. +T: plus tacrolimus treatment; +S: plus sirolimus treatment.

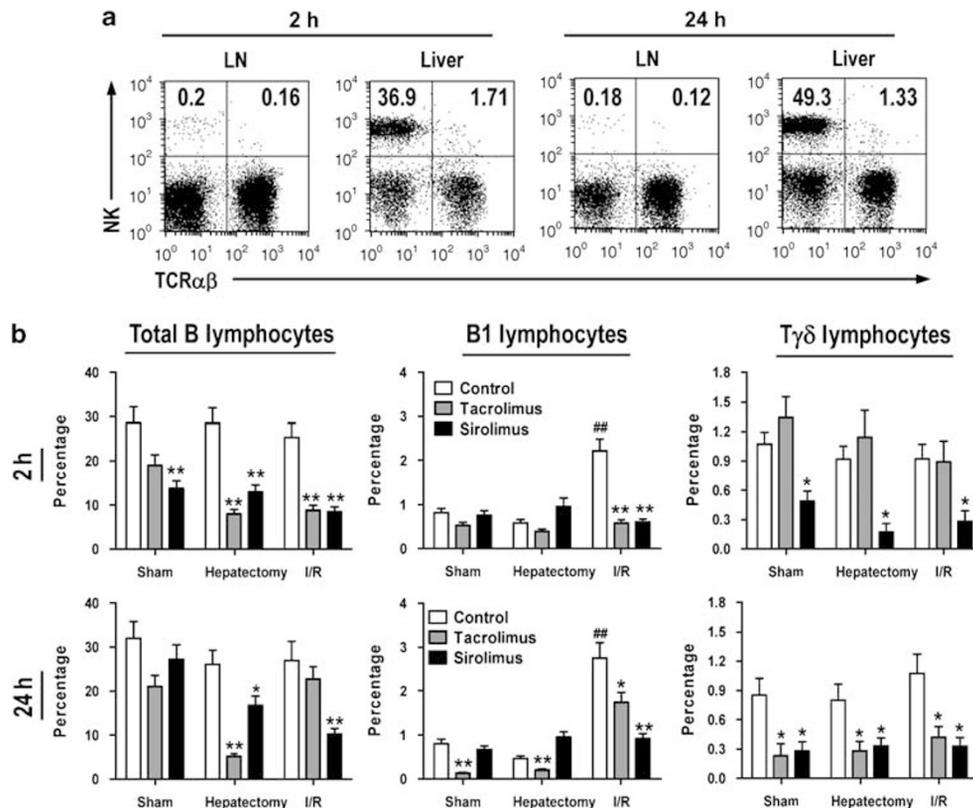


Figure 5 Changes in NK cells, and B and T $\gamma\delta$ lymphocyte populations, isolated from liver samples. Different leukocyte subpopulations, obtained from lymph node (LN) and liver of the different groups of rats as indicated, were analyzed by cytofluorimetric analysis of NK cells, and B and T $\gamma\delta$ lymphocyte markers. (a) The dot plots represent the NKR-P1A and TCR $\alpha\beta$ expression on gated lymphocytes from LN and liver of sham control rats killed at 2 and 24 h as indicated, exhibiting a higher proportion of classical NK (NKR-P1A⁺ TCR $\alpha\beta$ ⁻) and NKT (NKR-P1A⁺ TCR $\alpha\beta$ ⁺) cells in liver parenchyma. (b) Proportions of total B (OX12⁺) and B1 (OX12⁺ CD5⁺), and TCR $\gamma\delta$ lymphocytes from the liver of different groups of rats killed at 2 and 24 h, as indicated. The graphs represent the mean and s.e. of values obtained from isolated hepatic lymphocytes of at least six animals, and they evidence an effect of both drugs in decreasing total B and T $\gamma\delta$ lymphocytes, and in reverting the ischemia/reperfusion (I/R)-induced increase of B1 cells. * $P < 0.05$ vs its control; ** $P < 0.01$ vs its control; ## $P < 0.01$ vs other untreated controls. +T: plus tacrolimus treatment; +S: plus sirolimus treatment; h: hours.

hepatectomy and I/R groups did not result in local or systemic changes in the TCR $\gamma\delta$ proportion.

In a variety of conditions, including organ transplantation,²⁴ a premature exit of the thymus of DP cells has been reported; however, the presence of these cells in our model was small, discarding changes in the hematothymic barrier.¹⁰ We also ruled out an augmented extrathymic T-cell differentiation by determining TCR⁺ CD8 $\alpha\alpha$ lymphocytes, whose peripheral proportion had been reported to increase in some pathological situations, including liver injury.²⁵

In this study, both tacrolimus and sirolimus showed a beneficial effect by reducing the mortality of I/R animals. The sirolimus dose (2 mg/kg) was confined within the range employed experimentally for immunosuppression or graft tolerance in rats. When administered as a single dose, rodent studies have shown a dose–response relationship with allograft survival. Sirolimus doses of 2–6 mg/kg/day provided maximum graft survival in the heterotopic heart allograft model,²⁶ and intraperitoneal doses as high as 24 mg/kg have been used to induce tolerance to skin allograft in mice.^{27,28}

The tacrolimus dose employed by us (6 mg/kg) was also near or within the range that has been used in experimental models of allograft rejection^{29–31} or of the injury induced after reperfusion³² or partial hepatectomy.³³ In addition, some studies suggest that a higher than usual dose of tacrolimus is required for inhibiting the innate cell-mediated immune response, and doses of 10 mg/kg/day have been used in mice for this purpose.^{34,35} Yet, it is difficult to judge whether both drugs have been administered in equivalent doses, because of the large amplitude of the dosage range used in animal models, their different mechanism of action and their uneven spectrum of collateral effects.

The protective effects of tacrolimus and sirolimus observed here were accompanied by a decrease, not only in the area of necrosis, but also in the percentage of apoptotic cells. Necrosis is typically the consequence of an acute metabolic disturbance, whereas apoptosis represents the execution of a programmed sequence, often triggered by a specific stimulus. Both TNF α ³⁶ and IL-4³⁷ have been shown to trigger hepatocyte apoptosis. Furthermore, these and other mediators can

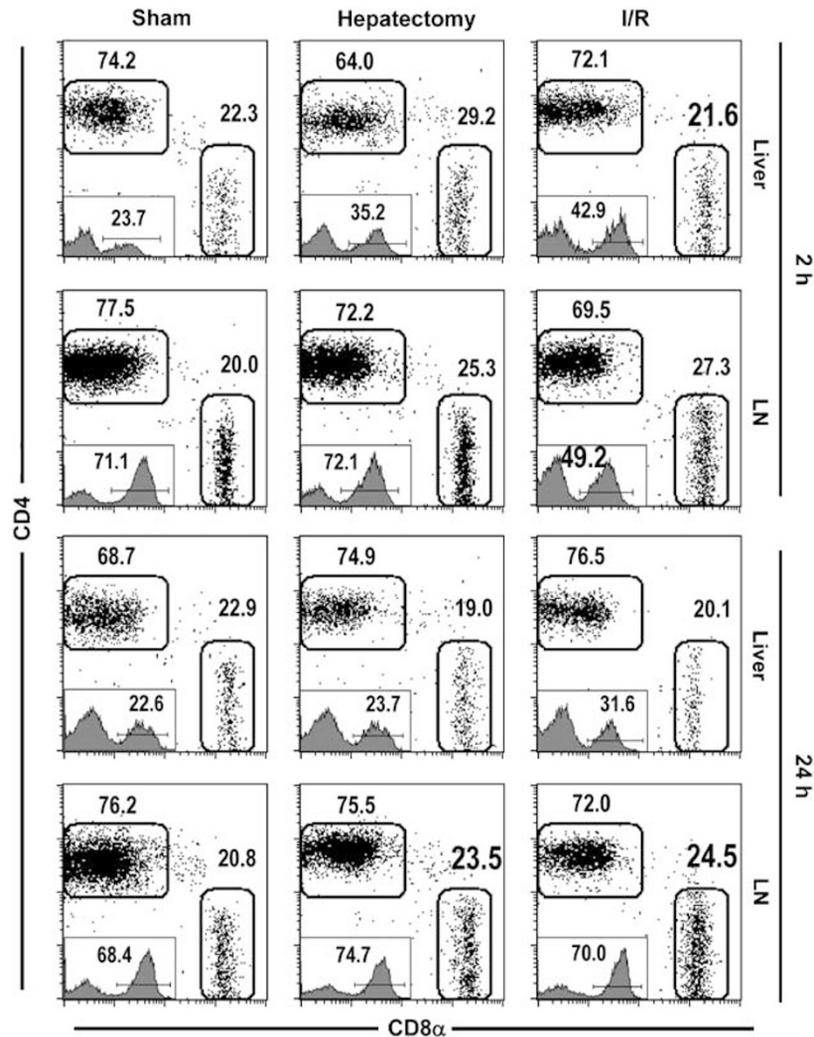


Figure 6 T lymphocyte proportion underwent a transient increase in the liver after ischemia/reperfusion (I/R). The proportion of lymphocyte T $\alpha\beta$ subpopulations was determined by flow cytometry. CD4 vs CD8 α expression (dot plots) was analyzed on gated TCR $\alpha\beta$ cells (histograms) from liver and lymph node at different intervals after reperfusion as indicated. A discrete shift in the proportion of T cells from the lymph node to the liver, without changes in CD4 or CD8 subpopulations, is appreciated in I/R groups at 2 h. Data on the plots are representative of at least six independent experiments.

be released by infiltrating lymphocyte subpopulations, some of which could also upregulate their Fas-L or TLR-2 expression, which are also known to trigger hepatocyte apoptosis.³⁸ Therefore, apoptosis might be a more sensitive parameter than necrosis in measuring the effect of immunosuppressive drugs, otherwise supposed to exert their action primarily on lymphocytes. The distinction between necrosis and apoptosis can be in fact artificial, as the necrosis, in certain circumstances, can be considered to be an abortive apoptosis because of their being insufficient ATP to complete the apoptotic program.³⁹ Molecules with known mitochondrial protective capacity, including tacrolimus,⁴⁰ also protect against apoptosis.⁴¹ I/R plus hepatectomy is a particularly energy-demanding situation, and preventing mitochondrial dysfunction may contribute to the beneficial effect of tacrolimus, especially when this effect seems to be independent of the immunosuppressive action.⁴² In contrast,

sirolimus, by activating procaspase-3, can induce apoptosis,⁴³ which may explain the slight apoptosis increase we found at 24 h (data not shown), although the protective properties of sirolimus seem to prevail at 2 h.

The stimulating effect of tacrolimus and other calcineurin inhibitors on hepatic regeneration is well known,^{44,45} although the mechanism is far from clear.^{44,45} On the other hand, our and others' results show an abundance of NK and NKT cells in the liver relative to other lymphoid organs.⁴⁶ These cells exert a toxic effect on cycling hepatocytes, resulting in inhibition of liver regeneration as a net effect.^{44,47} Therefore, a possible hypothesis is that the inhibitors of calcineurin could operate through the inhibition^{44,45} or elimination⁴⁸ of intrahepatic NK cells. Tacrolimus can inhibit the expansion of NKT cells in mice undergoing partial hepatectomy,³³ and our results show a decreased NK cells liver infiltrate in tacrolimus-treated groups. However,

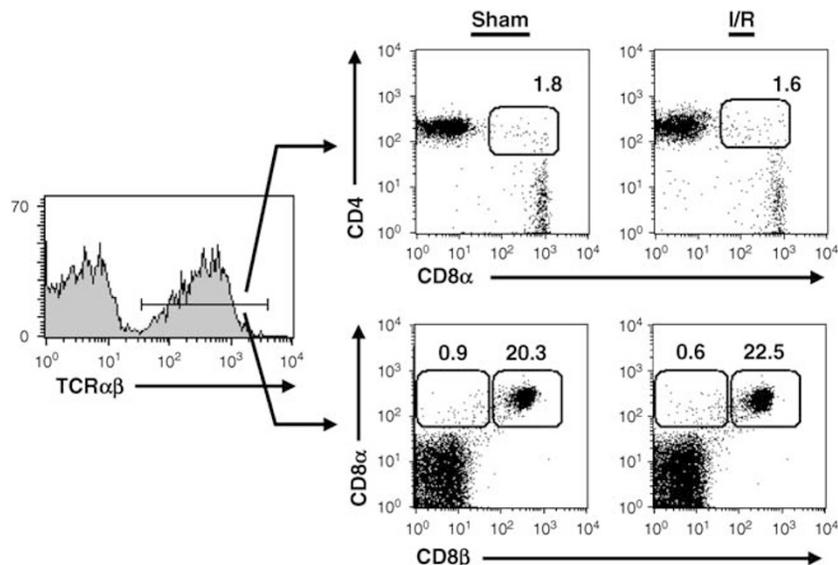


Figure 7 Double-positive and CD8 $\alpha\alpha$ T lymphocytes are almost absent in the liver. Intrahepatic lymphocytes of different group rats, killed at 2 and 24 h, were stained with anti-TCR $\alpha\beta$, anti-CD4, anti-CD8 α and anti-CD8 β antibodies for cytofluorimetric analysis. The left histogram shows the expression of TCR $\alpha\beta$ in the total hepatic lymphocyte population. The dot plots represents the CD4 vs CD8 α (upper row) and the CD8 α vs CD8 β (lower row) expression among TCR $\alpha\beta$ ⁺ lymphocytes, showing that double-positive (CD4⁺CD8 α ⁺) or CD8 $\alpha\alpha$ (CD8 α ⁺CD8 β ⁻) T lymphocytes neither reach important proportions nor change after ischemia/reperfusion (I/R). Data on the plots are representative of at least six rats of the indicated groups killed at 24 h, and similar plots were obtained from rats of the remaining groups.

sirolimus, which inhibited regeneration, also decreased NK cells, arguing against the hypothesis of an indirect, NK- or NKT-mediated, effect of tacrolimus in stimulating hepatocyte regeneration.

Both decrease of hepatic injury and increase of regeneration might be involved in the beneficial effect of tacrolimus. Sirolimus, however, induced a profound inhibition of hepatocyte regeneration. To force hepatocytes to divide can compromise the capacity of a limited residual hepatocyte mass to cope with increased metabolic demands. Thus, both stimulus and inhibition of the hepatic regeneration can be beneficial, but it seems more probable that this could be an effect of relatively minor importance for the outcome. Therefore, the decrease in mortality found in our model might well have been entirely because of alternative mechanisms of protection.

Our data show that a great number of T lymphocytes are recruited in the liver early after injury, further supporting a role for these cells in the early unspecific damage ensuing after I/R. The effect of immunosuppressants in reducing the intrahepatic number of T lymphocytes may have contributed to their protective effect, probably in part by decreasing PMN accumulation. Besides the known effect of these drugs on the proliferation of T cells, this decreased lymphocyte infiltrate might be partly because of the suppressing effect of calcineurin inhibitors on the endothelial adhesion of lymphocytes through the route VLA-4/VCAM-1.⁴⁹ Similar mechanisms could have contributed in treated rats to the observed decrease of B and T $\gamma\delta$ cells, although we cannot know their

relative contribution to the protective action of both drugs in the I/R injury.

The effector mechanisms underlying CD4⁺ T cell-mediated tissue injury are obscure. These lymphocytes could be activated by chemokines and cytokines released by other infiltrating cells, and proinflammatory molecules can drive the naïve T cells to a more aggressive phenotype.⁵⁰ CD4⁺ T lymphocytes have been functionally differentiated into Th1 cells that produce INF γ , lymphotoxin and TNF α , and Th2 cells that produce IL-4, IL-5, IL-6, IL-10 and IL-13.⁵¹ An inflammatory pattern of Th1 predominance seems pathogenic, whereas a polarized Th2 pattern may be protective against I/R injury.^{50,52} Although the precocity of the Th1/Th2 response in our model is noteworthy, a negative correlation between parameters of liver injury and expression of Th2 cytokines has been observed in humans already at 90 min after reperfusion of the liver.⁵³ Th1/Th2 changes disappeared in animals killed at 24 h, suggesting an acute, short-term effect. Neither were these changes mirrored in the submaxillary LN, indicating also a compartmentalization in the response; therefore, the ability we found for tacrolimus and sirolimus to antagonize the early increase in Th1/Th2 ratio could have been more important in reducing the hepatocellular injury than to modify the total number of CD4⁺ T cells.

Regulatory T lymphocytes can suppress the response not only of other effector lymphocytes,⁵⁴ but also of dendritic cells and macrophages,⁵⁵ and they may play a role in non-specific inflammatory reactions. A systemic amplification of

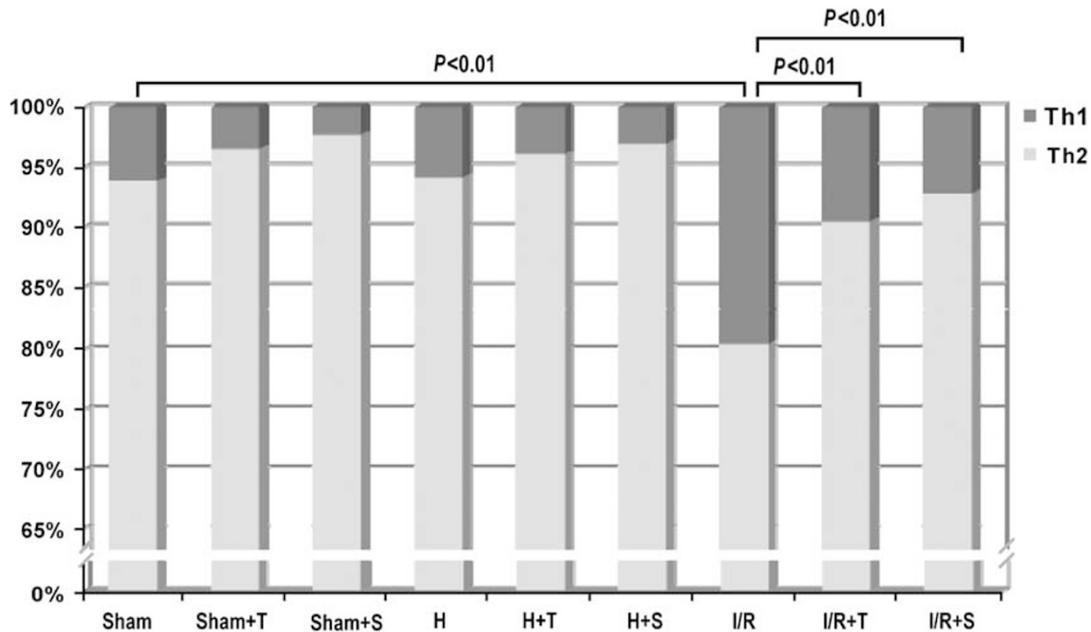


Figure 8 Liver parenchyma Th1/Th2 CD4 T cell proportions are modulated by immunosuppressive drugs. Liver $\text{TCR}\alpha\beta^+ \text{CD4}^+$ lymphocytes were analyzed for CD45RC expression by flow cytometry to determine their Th1 (CD45RC^{high}) or Th2 (CD45RC^{low}) phenotype. The relative proportions of Th1 and Th2 among CD4 T lymphocytes are represented in a stacked bar chart, which displays the mean values obtained from isolated hepatic lymphocytes of at least six animals in the different groups as indicated. There is a significant increase in the Th1/Th2 proportion after ischemia/reperfusion (I/R), which is partially reverted by both immunosuppressants. H: hepatectomy; +T: plus tacrolimus treatment; +S: plus sirolimus treatment.

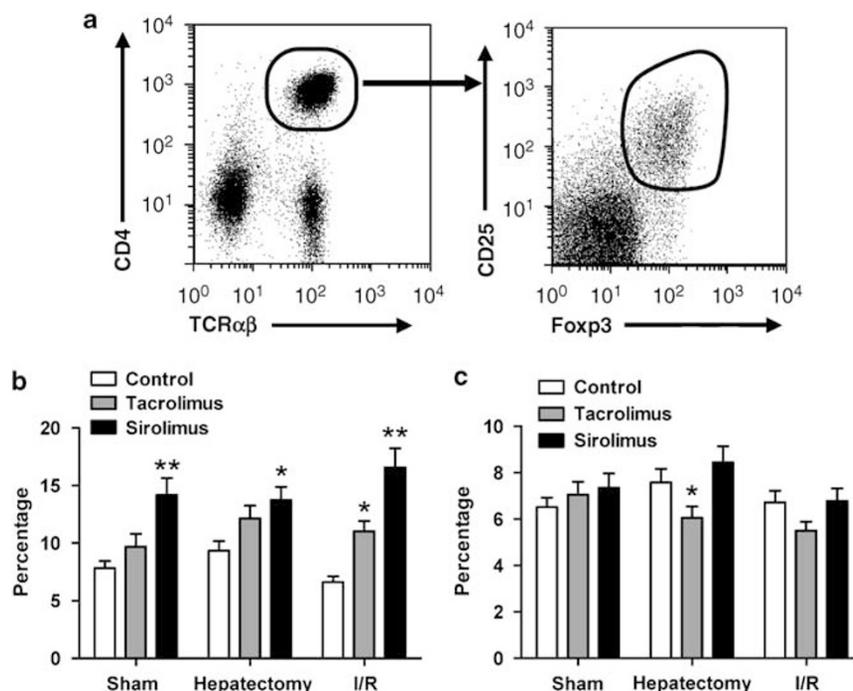


Figure 9 Local increase of regulatory T lymphocytes in the liver parenchyma. (a) The CD25 and Foxp3 markers were used to study the regulatory T lymphocytes in gated $\text{TCR}\alpha\beta^+ \text{CD4}^+$ population (right dot plot). The left dot plot shows a direct correlation between the expression of both markers, allowing us to define regulatory T lymphocytes as $\text{CD25}^+ \text{Foxp3}^+$ among the $\text{TCR}\alpha\beta^+ \text{CD4}^+$ population. (b, c) The bar graphs represent the proportion of regulatory T cells among the CD4 T lymphocytes of livers (b), or lymph nodes (LN) (c), at 24 h after reperfusion. Each bar in graphs represents the mean and s.e. of values found in at least six animals per group. * $P < 0.05$ vs control; ** $P < 0.01$ vs control. I/R: ischemia/reperfusion.

the Treg population has been reported after severe injury and it might contribute to the post-injury immunosuppression.⁵⁶ Sirolimus can expand peripheral CD4⁺CD25⁺ cells and their regulatory capacity *in vivo*,⁵⁷ as well as the selective survival of these cells *in vitro*.⁵⁸ As the specificity of location into the inflamed organ seems crucial for Treg cells to exert their function,⁵⁹ the increased proportion of CD4⁺CD25⁺Foxp3⁺ cells only in the liver adds further support to the hypothesis that sirolimus exerts its protective effect partly by this mechanism. In contrast, calcineurin inhibitors have been reported to induce a peripheral depletion in Treg cells,^{60,61} and to suppress their function.⁶² The reason for such a divergent effect could be the well-known differences between calcineurin inhibitors and m-TOR inhibitors regarding their action on IL-2,⁶² the signaling through this interleukin being crucial for the activity of CD4⁺CD25⁺Foxp3⁺ cells.⁶³ Tacrolimus decreased Treg cells at the LN in I/R rats, but, interestingly, their proportion was increased in the liver.

In summary, the protection achieved by both tacrolimus and sirolimus in hepatic I/R is accompanied by a decrease of hepatic T lymphocytes and by complex changes in the relative proportions of lymphocyte subpopulations. The net effect would be not only a decrease in the main effector cells, but also an increase of those lymphocyte subpopulations with regulatory activity—an early decrease in the Th1/Th2 balance and a progressive increase in the Treg lymphocytes. This suggests that, despite their different signaling pathways, both drugs modify the early-local immune response in a common way. It also opens a door to future studies of new therapeutic strategies against the I/R injury directed at harnessing lymphoid subpopulations with regulatory capability instead of the traditional strategy to neutralize/eliminate effector cells or their products.

ACKNOWLEDGEMENTS

We thank Angeles Vicente, Rosa Sacedón and Marina Guerin for their valuable suggestions, Pablo González and Cruz Rodríguez-Bobada for their help with the animal procedures and care, Carmen Hernández and Amalia Vázquez for their assistance with the cytometry analyses, and Cati Escribano and Virginia Peinado for their help with the H-E staining. This work was supported by grants BFU2004-03132, BFU2007-65520, GR74/07-910552 and RD06/0010/0003 from the Spanish Government.

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