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Strain-specific in vitro cytokine production profiles do not predict rat liver allograft survival

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Abstract The aim of this study was to assess whether differences in cytokine production between inbred rat strains could explain differences in liver allograft survival. Splenocytes from five different strains were cultured with Concanavalin A to determine in vitro cytokine production profiles. Strain-specific TNF-α, IFN-γ, IL-6 and IL-10 responses in naive animals were not associated with survival after rat liver transplantation. To investigate whether in vitro cytokine responses changed during the allogeneic inflammatory response, Brown Norway livers were transplanted to Lewis and Pivold Virol Glaxo recipients. During the early postoperative phase IL-6 and IL-10 (Th2-like) responses were significantly up-regulated in Lewis recipients, whereas Th2-like responses were not increased in Pivold Virol Glaxo. Our results do not support the generally held view that differential in vitro cytokine responses are related to liver allograft survival but suggest that cytokine responses are affected by the allogeneic inflammatory response after liver allografting.

Keywords Liver transplantation · Cytokine profiles · Splenocytes · Mitogens, · Allograft survival · Acute rejection

Introduction

Liver allografts can be spontaneously accepted in fully mismatched donor recipient combinations of rat inbred strains [1, 2]. In major histocompatibility complex (MHC)-identical strains August and Pivold Virol Glaxo (AUG and PVG, both $RT1^c$), AUG reject PVG livers in delayed fashion whereas in the reverse direction (AUG into PVG) the grafts show long-term survival [3]. This indicates that non-MHC genes in the recipient are

important in determining the strength of rejection. Possible candidates of genes determining the strength of rejection and liver allograft survival can be found in a range of cytokine genes.

Since differential cytokine production may explain differences in the outcome after allografting, polymorphisms in cytokine genes and in vitro production of cytokines have been investigated. In the rat IL-4 gene, a promotor region polymorphism has been identified which was associated with the level of IL-4 production in vitro [4]. In human studies, polymorphisms in several human cytokine genes have been related to higher or lower production of cytokines upon in vitro stimulation [5, 6, 7, 8]. Furthermore, some cytokine gene polymorphisms have been associated with the occurrence of human solid organ graft rejection [9, 10, 11, 12, 13]. More recently, differential levels of in vitro TNF-alpha production have also been associated with human liver allograft rejection [14, 15]. In vitro cytokine responses can be affected by factors such as age, underlying disease and immunosuppressive therapy [16, 17, 18]. These factors may enhance or negate the relevance of cytokine production profiles with respect to allograft rejection. To avoid these possible confounding factors, we used an animal model to study strain-specific in vitro cytokine responses in relation to liver allograft survival.

Zimmerman et al. [3] reported that inbred rat strains exhibit remarkable differences in survival after liver transplantation. For example, Dark Agouti (DA), Lewis (LEW) and PVG recipients spontaneously accept liver allografts across a complete allogeneic barrier without the need for treatment to promote acceptance, whereas Brown Norway (BN) and AUG reject liver allografts in the short-term. Differences in strain-specific cytokine production profiles may influence the severity of the allogeneic inflammatory response after liver allografting. Therefore, we hypothesize that strain-specific in vitro cytokine production profiles could predict survival after liver allografting in the rat. To test this hypothesis, we determined the endogenous capacity of lymphocytes to produce Th1- (TNF-α, IFN-γ) and Th2- (IL-6, IL-10) type cytokines upon a standardized in vitro stimulus in five different naive inbred rat strains (AUG, BN, DA, LEW and PVG).

The generally held view is that cytokine production profiles determine the severity of inflammation, although it is also possible that cytokine production levels are affected by the allogeneic inflammatory response. Therefore, we also tested the hypothesis that strain-specific in vitro cytokine production profiles are affected by the inflammatory response after liver allografting. For this purpose, long-term surviving BN livers were allografted to recipient strains with respectively high (LEW) and low (PVG) cytokine responses in naive animals.

Materials and methods

Animals

Pathogen-free male inbred AUG/OlaHsd (RT1^c), BN/RijHsd (RT1^n), DA/OlaHsd (RT1^a), LEW/HanHsd (RT1^l) and PVG/OlaHsd (RT1^c) rats were obtained from Harlan (The Netherlands). Animals of all strains weighed approximately 250 g (12 weeks of age). They were kept under specific pathogen-free conditions and fed a standard pellet diet and water ad libitum. The experimental protocols adhered to the rules in the "Dutch Animal Experimentation Act" (1977) and the published "Guidelines on the Protection of Experimental Animals" by the Council of the European Commission (1986). The Committee on Animal Research of the Erasmus University, Rotterdam, The Netherlands, approved the specific protocols.

Liver transplantation

Orthotopic liver transplantation was performed by means of the technique described by Kamada and Calne [19], using the cuff technique without hepatic artery revascularization. In brief, the donor liver was flushed by the portal vein and hepatic artery with a hypotonic citrate buffer solution through a cannula placed in the aorta. After recipient hepatectomy the suprahepatic vena cava was anastomosed with a 8–0 vascular suture. The portal vein and infrahepatic vena cava cuffs were

Table 1 Liver allograft survival between different inbred rat strains

Recipient strain (MHC)	Donor strain	Liver graft survival	Reference
AUG (RT1^c)	BN	Delayed	[3]
AUG (RT1^c)	DA	Short	[3]
AUG (RT1^c)	LEW	Short	[3]
AUG (RT1^c)	PVG	Delayed	[3]
BN (RT1^n)	AUG	Delayed	[3]
BN (RT1^n)	DA	Short	[3]
BN (RT1^n)	LEW	Delayed	[3, 20]
BN (RT1^n)	PVG	Delayed	[3]
DA (RT1^a)	AUG	Long	[3]
DA (RT1^a)	BN	Long	[3]
DA (RT1^a)	LEW	Long	[3, 22, 23]
DA (RT1^a)	PVG	Long	[1]
LEW (RT1^l)	AUG	Short	[3]
LEW (RT1^l)	BN	Long	[3, 20] and this study
LEW (RT1^l)	DA	Short	[3, 23, 21]
LEW (RT1^l)	PVG	Short	[3, 24]
PVG (RT1^c)	AUG	Long	[3]
PVG (RT1^c)	BN	Long	[3] and this study
PVG (RT1^c)	DA	Long	[3, 20]
PVG (RT1^c)	LEW	Delayed	[3]

then secured with a free ligature. The bile duct was rejoined by end-to-end anastomosis over an internal stent. The portal vein was clamped for less than 20 min in all animals.

Eight LEW ($RT1^l$) and 8 PVG ($RT1^c$) rats were used as recipients of BN ($RT1^n$) liver grafts and were killed on postoperative day 7 and 21, four animals per time point. The transplanted livers were removed for histology along with the spleen for isolation and culture of lymphocytes. In addition, two animals of both recipient strains were killed on postoperative day 100 to confirm spontaneous long-term survival of BN liver allografts as described previously [3, 20].

Liver allograft survival

We summarized liver allograft survival, as reported previously [1, 3, 20, 21, 22, 23, 24], to assess a possible relationship between the capacity to produce cytokines upon in vitro stimulation and liver allograft survival without immunosuppression (Table 1). When recipients of liver allografts survived for more than 100 days, they were classified as long-term survivors. Of the five different recipient strains summarized in Table 1, long-term graft survival was observed in DA, LEW and PVG, but LEW only showed long-term graft survival when BN donors were used. AUG and BN displayed rejection of all liver allografts in the short-term (median survival less than 30 days) or in the long-term (more than 30 days).

Histopathology

Tissues from liver allografts obtained at postoperative day 7 and 21 were fixed in buffered formalin. Paraffin-embedded tissues were sectioned and stained with hematoxylin and eosin. All sections were scored in a blind fashion by a pathologist (P. E. Zondervan) according to the Banff criteria for grading liver allograft rejection, including portal inflammation, bile duct inflammation or damage and venous endothelial inflammation [25].

Isolation of lymphocytes from spleen

Spleens from naive animals (AUG, BN, DA, LEW, PVG) were minced and mashed through a fine sieve in phosphate-buffered saline. Mononuclear leucocytes were isolated from red blood cells and polymorphonuclear leucocytes by centrifugation over a Ficoll gradient (Ficoll-Paque Research Grade, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Cells collected from the interface were washed twice, taken up in RPMI 1640 medium (Dutch modification, Gibco, Life Technologies, Paisley, Scotland) supplemented with 3 mM L-glutamine (Gibco) and 10% foetal calf serum and stored overnight at 37°C in a humidified atmosphere containing 5% CO_2. Lymphocytes from spleens of LEW and PVG at postoperative day 7 and 21 were isolated according to the same protocol and frozen with 10% DMSO and 20% foetal calf serum in liquid nitrogen before culture.

In vitro cytokine production

After overnight storage in RPMI 1640 medium (Gibco) at 37°C or after thawing from liquid nitrogen, viable splenocytes from naive ($n=7$) and allografted animals ($n=8$), respectively, were counted using trypan blue exclusion. Subsequently, splenocytes (mainly lymphocytes) were cultured in RPMI 1640 medium with L-glutamine and 10% foetal calf serum with 10 μg ml^{-1} Concanavalin A (Con A, Sigma Biosciences St. Louis, Mo., USA) at 5×10^5 ml^{-1} in 100 μl replicates in 96-well U-bottomed plates (Costar tissue culture treated polystyrene, Corning, N.Y., USA). Initially, five replicate cultures were set up to harvest culture supernatants at days 1, 2, 3, 4 and 7 after onset of cell culture. Furthermore, lymphocytes from naive animals (3 animals per strain) were cultured with Con A (10 μg ml^{-1}), bacterial lipopolysaccharide (10 ng ml^{-1}) and phytohemagglutinin (5 μg ml^{-1}) to evaluate the effect of different in vitro stimuli. Culture supernatants were stored at −20°C until cytokines were measured. After thawing culture supernatants, IL-6, IL-10, IFN-γ and TNF-α levels were determined using commercially available ELISA assays (Cytoscreen Immunoassay Kit, Biosource International, Camarillo, Calif., USA) according to the manufacturer's instructions. Cytokine concentrations were calculated by reference to the manufacturer's supplied cytokine standards and expressed in picogrammes per millilitre.

Flow cytometry

To assess a possible correlation between in vitro cytokine responses and the spleen cell composition of different inbred rat strains, the percentages of viable monocytes, B and T lymphocytes were determined by flow cytometry using different specific antibodies. Splenocytes (10^5 cells) were incubated (30 min, 4°C) with mouse monoclonal antibody ED1, recognizing monocytes and macrophages [26] and OX19, an anti-rat CD5 monoclonal antibody, exclusively expressed on T cells (Serotec, Oxford, UK). Biotinylated rabbit anti-rat IgM/IgG polyclonal antibodies were used to identify B lymphocytes (The Jackson Laboratory, Bar Harbor, Me., USA). Antibody binding was detected using

Table 2 Classification of inbred rat strains into (relatively) high, intermediate (*Int*) or low cytokine producers (7 animals per strain)

Strain	TNF-α[a]	[TNF-α] day 3 (pg/ml)[b]	*P*	IFN-γ[a]	[IFN-γ] day 3 (pg/ml)[b]	*P*	IL-6[a]	[IL-6] day 3 (pg/ml)[b]	*P*	IL-10[a]	[IL-10] day 3 (pg/ml)[b]	*P*
AUG	Low	14.1 ± 6.7	0.008	Low	21.9 ± 8.6	1.000	Low	60.9 ± 52.1	0.795	Int	49.1 ± 10	0.066
BN	Int	93.9 ± 21.6	0.566	High	224 ± 79	0.050	Low	37.9 ± 24.7	0.709	High	277 ± 150	0.003
DA	High	293 ± 38.1	0.005	High	437 ± 200	0.006	High	105 ± 50.6	0.013	High	240 ± 157	0.001
LEW	Int	167 ± 42	0.417	High	317 ± 154	0.077	High	103 ± 21.5	0.017	High	137 ± 30	0.017
PVG	Int	227 ± 114		Low	39.9 ± 11.1		Low	24.7 ± 9.7		Low	32.2 ± 9.3	

[a]Mean TNF-α, IFN-γ, IL-6 or IL-10 production was classified as high or low when mean cytokine production levels differed significantly from strains with respectively lower or higher levels
[b]To illustrate the classification into high, intermediate and low responders, cytokine production levels at day 3 of cell culture are shown. Cytokine production levels at days 2, 4 and 7 of culture showed the same picture, whereas at day 1 of cell culture differences between inbred strains were not yet manifested

phycoerythrin conjugated rabbit anti-mouse IgG (Becton Dickinson, San Jose, Calif., USA) or streptavidin-PE (Caltag, Burlingame, Calif., USA). The percentage of positive cells was measured using FACScan flow cytometry (Becton Dickinson, Mountain View, Calif., USA). Dead cells were excluded from analysis based on staining with 7-amino-actinomycin D viability probe (BD Pharmingen, San Diego, Calif., USA). Isotype antibodies were included as controls.

Statistical analysis

Differences in mean cytokine (IL-6, IL-10, IFN-γ, TNF-α) production measured in supernatants during the consecutive days after onset of cell culture, between different strains were analysed by repeated measurements analysis of variance. For this analysis all cytokine levels were transformed logarithmically to obtain approximate normal distributions. The PROC MIXED program from the SAS-package (version 6.12) was used for analysis of variance. Pair-wise comparisons between strains were done only if the overall test was significant. The relationship between relative numbers of lymphocytes/monocytes and in vitro cytokine responses were evaluated by Spearman's rank correlation (SPSS version 10). All results are expressed as mean values ± standard error of the mean.

Results

In vitro cytokine production profiles of naive rat inbred strains

Mean cytokine levels during the period of culture (1 to 7 days) in response to Con A stimulation were compared between naive animals from AUG, BN, DA, LEW and PVG strains (7 animals per strain). Analysis of variance showed that the mean level of TNF-α production during the culture period was significantly higher in DA than in other strains (Table 2). AUG had significantly lower levels of TNF-α production than BN, LEW and PVG (intermediate TNF-α producers). AUG and PVG (low producers) had significantly lower levels of in vitro production of IFN-γ than BN, LEW and DA (high producers). DA and LEW produced significantly higher levels of IL-6 than other strains. PVG produced significantly lower levels of IL-10 than BN, DA and LEW, whereas AUG showed intermediate production of IL-10. When the effect of different in vitro stimuli was evaluated, only DA showed higher TNF-α and IL-6 responses upon Con A stimulation and BN produced higher levels of IFN-γ in response to Con A compared with lipopolysaccharide (LPS) and phytohaemagglutinin (PHA) stimulation. There were no further differences in cytokine responses upon Con A, LPS or PHA stimulation (data not shown).

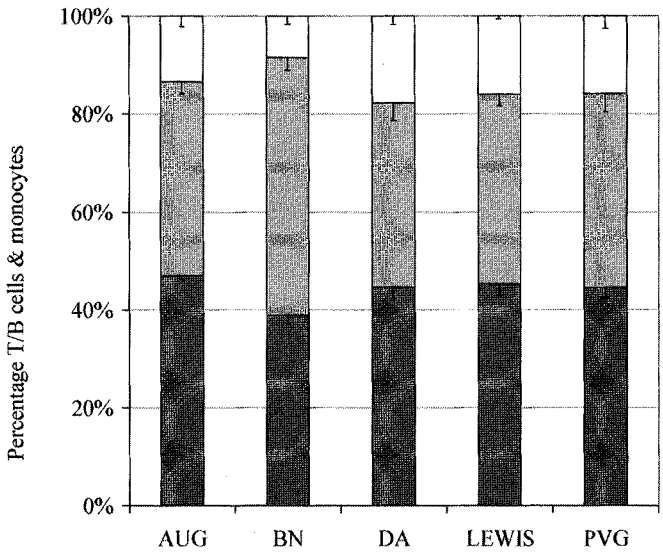


Fig. 1 Relative percentage of monocytes (±), B (±) and T (±) lymphocytes isolated from spleens of naive animals (7 animals per strain) of different rat inbred strains

Table 3 Banff grading of liver allografts, obtained at postoperative days 7 and 21, from LEW and PVG recipients of BN liver allografts. Mean value is in parentheses

Time point	LEW	PVG	P^a
Day 7	6, 6, 8, 9 (7.25)	6, 6, 7, 9 (7.0)	1.000
Day 21	5, 6, 6, 8 (6.25)	7, 8, 8, 8 (7.75)	0.143
Day 100	1, 2 (1.5)	2, 2 (2)	–

[a]Mann–Whitney U test

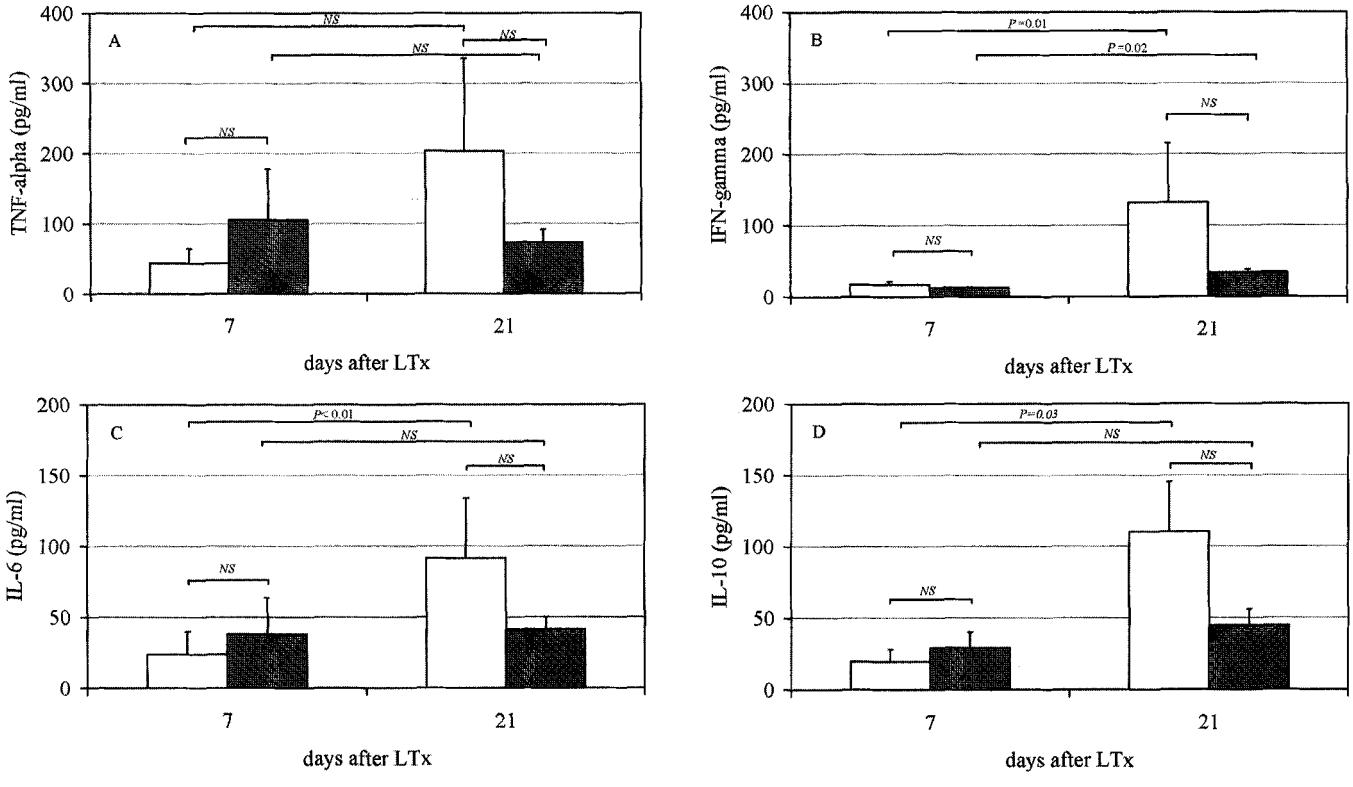
Composition of splenocytes

To assess whether differences in cytokine responses upon in vitro stimulation were influenced by the relative number of different cell types, splenocytes from naive animals were analysed by flow cytometry. There were only small differences in the relative number of monocytes, B and T lymphocytes isolated from spleens of different rat inbred strains (Fig. 1). When percentages of monocytes, B and T cells were related to in vitro cytokine responses, there were no significant correlations with TNF-α, IFN-γ, IL-6 or IL-10 production.

In vitro cytokine production profiles after liver allografting

To investigate whether in vitro cytokine production profiles of high (LEW) and low (PVG) responder strains changed during the early course after liver transplantation, we used LEW and PVG recipients as both strains accept BN livers despite differences in naive cytokine production profiles. When sections of liver allograft tissue were scored according to Banff criteria for grading liver allograft rejection, all animals showed moderate to severe acute rejection (Banff score 5 to 9) at postoperative day 7 and 21 and there were no significant differences in rejection grade between LEW and PVG (Table 3). In accordance with in vitro TNF-α production by naive animals, LEW and PVG did not differ in TNF-α levels when production was tested at postoperative day 7 and 21 (Fig. 2a). For the Th1-type cytokine IFN-γ, production was significantly up-regulated at postoperative day 21 compared with day 7 ($P \leq 0.02$) in both strains. Although mean postoperative IFN-γ levels (day 21) were higher in LEW than PVG, statistical sig-

Fig. 2a–d Post-transplantation in vitro cytokine responses in LEW (±) and PVG (±) recipients of BN liver grafts. Mean cytokine production after 3 days of culture with a standardized Concanavalin A stimulus is shown. **a** LEW and PVG did not show a significant up-regulation of TNF-α levels from postoperative days 7 to 21. **b** IFN-γ production was significantly up-regulated at postoperative day 21 compared with day 7 ($P \leq 0.02$) in both strains. **c** LEW showed significant up-regulation of IL-6 at postoperative day 21 ($P < 0.01$), whereas IL-6 was not significantly increased in PVG. **d** LEW showed significant up-regulation of the IL-10 response at postoperative day 21 compared with day 7 ($P = 0.03$)



nificance was not reached ($P=0.16$, Fig. 2b). Concerning the Th2-type cytokines IL-6 and IL-10, higher production of IL-10 at postoperative day 21 by the LEW strain did not reach statistical significance ($P=0.13$). When cytokine production was compared between timepoints after liver transplantation, LEW showed significant up-regulation of IL-6 and IL-10 at postoperative day 21 ($P<0.01$ and $P=0.03$, respectively), whereas IL-6 and IL-10 were not significantly increased in PVG (Fig. 2 c, d). Post-transplant cytokine responses in allografted animals were determined using nitrogen stored splenocytes, resulting in slightly lower responses as compared to cytokine production levels of freshly isolated splenocytes from naive animals (data not shown). The lowered response (due to freezing) precludes the comparison of post-transplant cytokine responses with pre-transplant cytokine production levels. In summary, although PVG showed low to intermediate cytokine responses in naive animals, we found a small but significant up-regulation of the IFN-γ response from postoperative day 7 to 21. In LEW we found up-regulation of both Th1- (IFN-γ) and Th2-type (IL-6 and IL-10) cytokine responses upon in vitro stimulation soon after liver transplantation.

Discussion

Results from this study show that naive strain-specific in vitro cytokine production profiles do not predict allograft survival after rat liver transplantation. For example, DA and PVG, both showing long-term graft survival, produced respectively high and low levels of IFN-γ, IL-6 and IL-10. Furthermore, AUG and BN, both rejecting liver allografts in the short-term or in the long-term, produced lower and higher levels of TNF-α, IFN-γ and IL-10, respectively. This observation does not confirm the role of cytokine production profiles in naive animals as promoters of the immune response after liver allografting. A possible explanation for this finding is that naive cytokine responses are affected by the allogeneic response after liver transplantation.

To test this hypothesis post-transplant cytokine responses were studied in high (LEW) and low (PVG) responder strains. When BN donors were used, both strains showed spontaneous long-term survival and there were also no significant differences in the strength of rejection during the early postoperative course (Table 3). In LEW recipients of BN allografts, both Th1- (IFN-γ) and Th2-type (IL-6 and IL-10) cytokine responses were significantly up-regulated during the early postoperative course, whereas Th2-type responses were not increased in PVG with lower Th1-like responses than LEW (Fig. 2). These data indicate that the endogenous capacity of mononuclear cells to produce cytokines upon in vitro stimulation is modulated during the immune response after liver transplantation. Consequently, pre-transplant cytokine responses do not necessarily reflect the level of cytokine production after liver allografting.

Kamada et al. described that spontaneous liver allograft acceptance is not due to the absence of an immune response; there is clear histological evidence of acute rejection with mononuclear cell infiltration of portal tracts and sinusoids [27]. We also found histological evidence of moderate to severe rejection in LEW and PVG recipients of long-term surviving BN liver allografts. Moreover, a paradoxical early immune activation during the development of spontaneous liver allograft acceptance has recently been described [28] with an early up-regulation of pro-inflammatory cytokines in spleen and lymph nodes of the recipient [29]. In this study we cultured mononuclear cells isolated from spleens of LEW and PVG recipients of long-term surviving liver allografts. Therefore, we speculate that increased IFN-γ responses as observed in LEW and PVG liver allograft recipients reflect a higher activation-state of the Th1-type cell population.

To date, there have been no previous reports on cytokine production upon in vitro stimulation in different inbred rat strains. We evaluated whether these strain-specific in vitro cytokine production profiles were influenced by the type of in vitro stimulus or variation in the composition of cell types used for culture. For AUG, LEWIS and PVG, cytokine production profiles upon stimulation with Con A, LPS and PHA were comparable, whereas DA and BN seemed to produce higher levels of TNF-α, IL-6 and IFN-γ, respectively, upon stimulation with Con A (data not shown). We observed some variation in the composition of cell types isolated from spleens of different inbred strains (Fig. 1); however, the relative number of monocytes, B and T lymphocytes did not correlate with cytokine production levels. These data suggest that differences in cytokine production profiles between inbred rat strains are, at least in part, a consequence of strain-specific factors regulating the activity of cytokine genes.

In accordance with results from this animal study, human studies investigating pre- and post-transplant in vitro cytokine responses did not find an association between differential production of IL-10 and the occurrence of acute liver graft rejection [14, 15]. Since LEW and PVG did not differ in rejection grade and in vitro TNF-α responses, our results neither support nor contradict previous studies showing an association between higher in vitro production of TNF-α and the occurrence of acute human liver graft rejection [14, 15].

In conclusion, different inbred rat strains could be classified as high-, intermediate- or low-responders with regard to in vitro TNF-α, IFN-γ, IL-6 and IL-10 production since naive animals of different strains show differences in their capacity to produce cytokines upon a

standardized in vitro stimulus. Although, cytokine gene polymorphisms associated with higher or lower production of cytokines, have been related to acute rejection of heart, kidney and liver transplants [9, 10, 11, 12, 13], our results indicate that strain-specific cytokine production profiles do not predict rat liver allograft survival. This finding does not support the generally held view that higher or lower in vitro cytokine responses determine the severity of allogeneic inflammatory responses and survival after liver allografting. An important question is whether in vitro cytokine responses are a cause or a consequence of the allogeneic inflammatory response after liver allografting. This study provides an argument in favour of the latter, since in vitro cytokine production profiles were altered during the allogeneic response after rat liver transplantation.

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