Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Association between heat shock protein 70s and Toll-like receptor polymorphisms with long-term renal allograft survival

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Keywords
gene polymorphism, HSP70, ischemia, kidney transplantation, Toll-like receptors.

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Received: 6 July 2005
Revision requested: 5 August 2005
Accepted: 7 December 2005

doi:10.1111/j.1432-2277.2006.00265.x

Summary

Long-term renal allograft survival has not improved significantly in recent years and only a minority of grafts survives for more than 15 years. To evaluate the association between *HSPA1A* G(190)C, *HSPA1B* A(1267)G and *TLR4* A(299)G polymorphisms and allograft survival we analyzed DNA of patients with long-term renal graft function over 15 years (Tx15), consecutively transplanted recipients (Tx), patients with acute rejection and healthy controls. *HSPA1B* (1267)AA was less prevalent in Tx versus Tx15 ($P = 0.02$) and versus controls ($P = 0.004$). *HSPA1B* (1267)GG was more frequent in Tx versus Tx15 ($P = 0.005$) and versus controls ($P = 0.002$). *HSPA1B* (1267)G allele occurred more often in Tx versus Tx15 ($P = 0.03$), and versus controls ($P = 0.02$). *TLR4* (299)AG genotype prevalence was increased in Tx15 versus Tx ($P = 0.02$), while *TLR4* (299)G allele was more frequent in Tx15 versus Tx ($P = 0.02$). The increased frequency of *HSPA1B* (1267)AA and *TLR4* (299)AG genotypes in Tx15 group indicates that better cytoprotective functions in *HSPA1B* (1267)AA and reduced proinflammatory response in *TLR4* (299)AG carriers might have improved renal allograft survival.

Introduction

Despite improvements in short-term renal graft survival because of modern immunosuppressive regimes, long-term renal allograft outcome has not improved significantly in recent years and only a minority of grafts survives for more than 15 years. Ischemia/reperfusion injury (I/R) and prolonged cold ischemia during renal transplantation are thought to be major determinants of acute and chronic rejection episodes and continue to be a serious problem [1,2]. However, various other factors including immunological mechanisms, infections, side effects of immunosuppressive treatment should also be taken into account.

Expression of heat shock protein (HSP) 70 chaperone family members is induced by ischemia, reperfusion and surgical stress, as well as by cytokine release [3]. As all these forms of stress are of significant relevance in transplantation, an enhanced expression of HSP70s under these conditions seems very likely. The involvement of HSP70 chaperones in protection against I/R injury of the transplanted kidney has recently been demonstrated [4]. HSP70s act by refolding proteins, thereby limiting cellular injury and restoring protein function. Moreover, they take part in adaptive alloimmune responses by activating Toll-like receptors (TLR)-induced proinflammatory cytokine cascade [5].

The human genes encoding members of the HSP70 family are localized on chromosome 6p21.3; *HSPA1A* (constitutive form) and *HSPA1B* (inducible form) [6], while the *TLR4* has been mapped to 9q32-33 [7]. Besides stress factors, genetic polymorphisms of these

genes also influence the expression of HSP70s and TLR4 proteins. For *HSPA1A*, a bi-allelic G(190)C silent mutation within the 5′ UTR was reported [8], while decreased HSP72 mRNA expression was measured in homozygous carriers of G variants at position 1267 of the *HSPA1B* coding region [9]. Although few other single nucleotide polymorphisms have been reported for HSP70 genes, recent studies focused on the relevance of *HSPA1A* G(190)C and *HSPA1B* A(1267)G in different diseases.

For *TLR4*, a single functional polymorphism has been identified within exon 3, corresponding to an asparagine/glycine amino acid substitution at position 299, which results in attenuated inflammatory response and decreased cytokine release [10].

Previously we demonstrated that long-term renal allograft survival is also influenced by genetic polymorphisms: angiotensin-converting enzyme DD genotype was associated with better long-term renal graft function [11], while the methylenetetrahydrofolate reductase T677 allele had opposite effects [12].

Considering the influence of genetic factors and the central importance of HSP72 and TLR4 expression in the pathogenesis of renal allograft rejection, we investigated the association of genetic polymorphisms of HSP70 chaperone family members and the TLR4 receptor with long-term renal allograft survival.

Methods

Patients

The present study was conducted at the First Department of Paediatrics at Semmelweis University Budapest, Hungary, the Transplant Centre of the Institute for Clinical and Experimental Medicine, Prague, Czech Republic and the Department of Nephrology of the University Clinic Essen, Germany. All cases and controls were Caucasian and gave informed consent for taking blood sample for DNA analysis. The ethical committees of all institutes approved the procedures.

Tx15 group

Forty-eight patients with preserved kidney graft function over 15 years [age: 54.1 ± 11.0 years; male/female: 22/26] were enrolled into the study as the long-term survival group (Tx15). All these recipients received cadaveric transplantation and took part in regular checkups. The standard immunosuppression in this group consisted of azathioprine and prednisolone. Two patients received mycophenolate mofetil instead of azathioprine because of liver disease in the 13th and 15th year, respectively.

Tx group

The group included 105 consecutively transplantated recipients (Tx) between 1999 and 2000 [age: 54.8 ± 12.6 years; male/female: 73/32]. Eighty-eight patients received their first cadaver kidney graft, seven received second cadaver graft and 10 patients received their graft from living donors. The immunosuppressive protocol was based on a triple therapy with cyclosporin A, azathioprine and prednisolone. AR episodes were treated with methylprednisolone pulses and in a case of steroid resistance with monoclonal or polyclonal antibodies against T cells (OKT3 or ATG). All Tx patients had stable kidney graft function 12 months after transplantation (serum creatinine <250 μg/l).

Acute rejection group

A total of 21 patients, transplanted between 2000 and 2004 [age: 45.6 ± 12.5 years; male/female: 12/9], who had lost their kidney grafts because of biopsy-proven AR within the first posttransplant year formed the AR group. Twelve patients received their first cadaver kidney graft, six received second, one third cadaver graft and two patients received their graft from living donors. Triple immunosuppressive therapy in this group was similar to that in Tx patients.

Healthy subjects

The prevalence of *HSPA1A* G(190)C, *HSPA1B* A(1267)G and *TLR4* A(299)G genotypes were obtained by studying a random, age-matched, unrelated population of 131 healthy blood donors [age: 58.5 ± 11.3 years; male/female:71/60].

Clinical data

Medical records of the patients were systematically reviewed. Data on age, gender, blood pressure, serum creatinine, proteinuria, body mass index, plasma cholesterol and triglyceride levels, number of AR episodes, immunosuppressive regimen, panel reactive antibody (PRA) levels, human leukocyte antigen (HLA) match, donor age, cold ischemic time, time on dialysis, basic renal disease were collected and entered into a database.

Samples and genotyping

Blood samples taken for regular routine checkup were used for genotyping. Genomic DNA was extracted from whole blood by using the QIAamp® blood Mini kit (Qiagen, Hilden, Germany). Genotyping of *HSPA1A* and *HSPA1B* was performed as described previously [13].

For *TLR4* the DNA fragments were amplified in a reaction mix containing 10% 10x polymerase chain reaction (PCR) buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.4 μM of each

specific primer (F:GATCAACTTCTGAAAAAGCATTC CAC, R:GATTAGCATACTTAGACTACTACCTCCATG), 1.5 U recombinant *Taq* polymerase (Invitrogen, Budapest, Hungary), with the following PCR conditions: initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, ending with a final extension at 72 °C for 10 min and cooling to 4 °C in a Perkin-Elmer Thermo Cycler® (PE, Model 2400; Norwalk, CT, USA). The PCR products were digested at 37 °C overnight, with *Nco*I (Sigma Chemical Co., Budapest, Hungary). The cleavage products were electrophoresed on 3% agarose gel and stained with ethidium bromide.

Statistical analysis

The genotype distribution between Tx, Tx15, AR and control groups was analyzed with Fisher's exact test using the approximation of Wolf and followed by Holm's posthoc test. Clinical data of patients were compared by ANOVA followed by Mantel–Hanzel test. Data are given as mean ± SD. The level of significance was set at $P < 0.05$.

Results

To test whether *HSP70* and *TLR4* polymorphisms are associated with long-term renal allograft outcome, we analyzed blood samples from patients with preserved kidney graft function over 15 years (Tx15), from consecutively transplanted recipients (Tx), from patients with graft loss within the first posttransplant year because of AR and healthy controls by PCR-restriction fragment length polymorphism. We evaluated the genotype distribution (homozygous wild-type, heterozygous and homozygous mutant) and the allele frequencies for all three polymorphisms, which are presented in Tables 1 and 2. Genotype distribution and allele frequencies of all polymorphisms in transplant and control groups fulfilled the criteria of the Hardy–Weinberg equilibrium.

Genotype distribution and allele frequencies of HSPA1A G(190)C polymorphism

There was no significant difference in the genotype or allele frequencies of the *HSPA1A* G(190)C polymorphisms among any of the groups.

Genotype distribution and allele frequencies of HSPA1B A(1267)G polymorphism

The *HSPA1B* (1267)AA genotype was less frequent in Tx patients compared with controls [$P = 0.004$; odds ratios (OR): 3.4; confidence intervals (CI): 1.35–8.94] and the Tx15 group ($P = 0.02$; OR: 0.29; CI: 0.09–0.89). The *HSPA1B* (1267)GG genotype was more frequent in Tx patients compared with controls ($P = 0.002$; OR: 5.1; CI:

Table 1. Genotype distribution of *HSPA1A* G(190)C, *HSPA1B* A(1267)G and *TLR4* A(299)G polymorphisms in transplanted patients with (Tx15) long-term graft survival, with acute rejection (AR) and in consecutively transplanted recipients (Tx) compared with healthy controls.

Polymorphism		*HSPA1A* G(190)C (%)			*HSPA1B* A(1267)G (%)			*TLR4* A(299)G (%)		
Genotype	*n*	GG	GC	CC	AA	AG	GG	AA	AG	GG
Control	131	2 (2)	8 (6)	121 (92)	24 (18)*	93 (70)	14 (12)†	120 (92)	11 (8)	0 (0)
Tx	105	0 (0)	5 (5)	100 (95)	6 (6)‡	81 (77)	18 (17)§	101 (96)	4 (4)¶	0 (0)
Tx15	48	0 (0)	6 (12)	42 (88)	9 (18)	36 (76)	3 (6)	41 (86)	7 (14)	0 (0)
AR	21	0 (0)	3 (14)	18 (86)	3 (14)	13 (62)	5 (24)	21 (100)	0 (0)	0 (0)

*$P = 0.004$ versus Tx in AA versus AG (OR: 3.4; CI: 1.35–8.94).
†$P = 0.002$ versus Tx in AA versus GG (OR: 5.1; CI: 1.65–16.0).
‡$P = 0.02$ versus Tx15 in AA versus AG (OR: 0.29; CI: 0.09–0.89).
§$P = 0.005$ versus Tx15 in AA versus GG (OR: 0.11; CI: 0.02–0.55).
¶$P = 0.02$ versus Tx15 (OR: 4.31; CI: 1.19–15.25).

Table 2. Allele frequencies of *HSPA1A* G(190)C, *HSPA1B* A(1267)G and *TLR4* A(299)G polymorphisms in transplanted patients with (Tx15) long-term graft survival, with acute rejection and in consecutively transplanted recipients (Tx) compared with healthy controls.

Polymorphism		*HSPA1A* G(190)C (%)		*HSPA1B* A(1267)G (%)		*TLR4* A(299)G (%)	
Allele frequencies	*n*	G	C	A	G	A	G
Controls	131	12 (4.5)	250 (95.5)	141 (54)	121 (46)*	251 (96)	11 (4)
Tx	105	5 (2)	205 (98)	93 (44)	117 (56)†	206 (98)	4 (2)‡
Tx15	48	6 (6)	90 (94)	54 (56)	42 (43)	89 (92)	7 (8)
AR	21	2 (5)	42 (95)	19 (45)	23 (55)	42 (100)	0 (0)

*$P = 0.024$ versus Tx (OR: 1.46; CI: 1.01–2.11).
†$P = 0.03$ versus Tx15 (OR: 1.61; CI: 0.99–2.63).
‡$P = 0.02$ versus Tx15 (OR: 4.531; CI: 1.156–14.19).

Table 3. *HSPA1A* G(190)C genotype distribution and clinical characteristics in transplanted patients with (Tx15) long-term graft survival, with acute rejection (AR) and in consecutively transplanted recipients (Tx) compared with healthy controls.

HSPA1A G(190)C	GG			GC			CC		
Groups	Tx	Tx15	AR	Tx	Tx15	AR	Tx	Tx15	AR
n	–	–	–	5	6	3	100	42	17
Males/females	–	–	–	3/2	3/3	2/1	70/30	19/23	11/7
Age (years)	–	–	–	53 [22–56]	53 [32–65]	61 [59–63]	53 ± 12	53 ± 11	42 ± 11
Creatinine (μmol/l)	–	–	–	113 [103–168]	126 [97–181]	390 [370570]	148 ± 48	145 ± 83	645 ± 215
Proteinuria (g/day)	–	–	–	1.6 [0.8–2.3]	0.7 [0.1–1.7]	2.2 [1.6–3.8]	1.1 ± 0.4	1.3 ± 0.7	2.9 ± 2.2
Chol (mmol/l)	–	–	–	5.2 [4.3–6.7]	4.9 [3.6–5.1]	ND	5.9 ± 1.2	5.9 ± 1.5	5.0 ± 1.7
TG (mmol/l)	–	–	–	2.0 [1.7–3.4]	2.3 [0.8–5.1]	ND	2.5 ± 1.3	1.9 ± 0.8	2.3 ± 0.9
BMI (kg/m^2)	–	–	–	26 [23–31]	23 [19–28]	23 [22–27]	27.0 ± 4.7	24.7 ± 3.6	24.0 ± 1
Hypertension, %	–	–	–	20	83	100	41	61	72
Acute rejection, %	–	–	–	19	16	100	31	28	100

Chol, cholesterol; TG, triglycerides; BMI, body mass index. Data are given as mean ± SD or in median ± range.

1.65–16.0) and the Tx15 group ($P = 0.005$; OR: 0.11; CI: 0.02–0.55).

The *HSPA1B* (1267)G allele occurred more frequently in the Tx group than in controls ($P = 0.024$; OR: 1.46; CI: 1.01–2.11) and the Tx15 group ($P = 0.03$; OR: 1.61; CI: 0.99–2.63).

Genotype distribution and allele frequencies of TLR4 A(299)G polymorphism

The prevalence of the *TLR4* (299)AG genotype was greater in Tx15 patients than in the Tx ($P = 0.02$; OR: 4.31; CI: 1.19–15.25) group. The*TLR4* (299)G allele occurred more frequently in the Tx15 group than in the Tx group ($P = 0.02$; OR: 4.531; CI: 1.156–14.19).

All *P* values remained significant after applying Holm's test.

Clinical characteristics of recipients

The clinical characteristics and demographic data of the study populations in *HSPA1A* G(190)C, *HSPA1B* A(1267)G and *TLR4* A(299)G polymorphism are included and compared in Tables 3, 4 and 5, respectively.

Discussion

Besides several other risk factors (e.g. I/R injury, infections, side effects of the immunosuppressive treatment), genetic predisposition is one of the most important determinants for late allograft loss. In the present study, we analyzed the influence of *HSPA1A* G(190)C, *HSPA1B* A(1267)G and *TLR4* A(299)G genetic polymorphisms on long-term renal allograft survival.

These genes play a crucial role during organ transplantation, inasmuch as the surgical procedure, warm and cold ischemia and also reperfusion induce the expression of HSP72 and TLR4 [4,5]. Furthermore, renal ischemia stimulates the expression of HSP72 during recovery of proximal tubular cells, and low levels of HSP72 are detrimental to renal recovery [14]. HSP70s are also assumed to protect cells from damage which may increase transplant survival [15]. In a kidney transplant model, graft survival correlated with the level of HSP72 expression after prolonged warm ischemia, which further underlines the role of HSPs during renal transplantation [16]. Recently, a close relationship has also been described between cellular protection represented by an improvement in the rate of graft survival and inducibility of HSP72 [17].

Although previous studies investigated the relevance of HSP70 polymorphisms in several ischemia-related situations and diseases [13,18,19], our data demonstrate for the first time an association between long-term renal allograft survival and genetic variations of HSPs and TLR4 in kidney transplantation.

We found that there are more (1267)AA carriers of *HSPA1B*, the gene of inducible HSP72 among patients with preserved graft function and healthy controls than in consecutively transplanted renal recipients. Moreover, *HSPA1B* (1267)GG genotype, which has been previously associated with impaired inducibility of HSP72 [9] was more frequent in the Tx group and tended to be more frequent in patients with graft loss because of AR than in Tx15 and control groups (Table 1). This raises the possibility that not only the decreased presence of *HSPA1B* (1267)AA, but also the increased frequency of the *HSPA1B* 1267(GG) genotype has a major impact on graft loss and allograft survival. The increased prevalence of the *HSPA1B* 1267(GG) genotype in patients with acute graft rejection further supports this assumption. However, one can also hypothesize that the presence of the G allele in

Table 4. *HSPA1B* A(1267)G genotype distribution and clinical characteristics in transplanted patients with (Tx15) long-term graft survival, with acute rejection (AR) and in consecutively transplanted recipients (Tx) compared with healthy controls.

HSPA1B A(1267)G	AA			AG			GG		
Groups	Tx	Tx15	AR	Tx	Tx15	AR	Tx	Tx15	AR
n	6	9	3	81	36	13	18	3	5
Males/females	3/3	6/3	2/1	58/23	16/20	5/7	12/6	0/3	3/3
Age (years)	36 [25–62]	48 [38–78]	46 [37–59]	53 ± 13	54 ± 10	45 ± 12	50 [24–63]	46 [37–62]	34 [22–63]
Creatinine (μmol/l)	100 [87–132]	118 [79–241]	600 [501–700]	147 ± 49	152 ± 86	607 ± 220	149 [104–253]	95 [88–164]	800 [650–1000]
Proteinuria (g/day)	1.2 [0.9–2.3]	1.3 [1.1–1.7	3.1 [1.7–4.7]	1.1 ± 0.4	1.6 ± 0.8	3.2 ± 2.1	1.3 [0.5–1.7]	0.6 [0.3–3.2]	ND
Chol (mmol/l)	1.2 [4.4–7.4]	6.3 [4.4–7.2]	3.8 [2.3–4.8]	6.0 ± 1.3	5.9 ± 1.6	6.0 ± 1.7	5.3 [3.8–7.5]	5.1 [4.8–6.8]	5.6 [2.8–6.3]
TG (mmol/l)	1.3 [0.9–3.4]	2.3 [1.2–3.1]	ND	2.5 ± 1.2	1.8 ± 0.8	2.5 ± 0.9	2.0 [1.1–4.2]	1.2 [1.1–1.3]	2.5 [1.6–3.5]
BMI (kg/m^2)	25 [19–31]	24 [20–27]	22 [20–29]	27.2 ± 5.2	24.7 ± 4	24 ± 1.0	27 [20–33]	24 [24–27]	22 [18–23]
Hypertension, %	33.3	77	30	32.9	75	90	33	44	66
Acute rejection, %	16.7	33	100	24.7	27	100	39	0	100

Chol, cholesterol; TG, triglycerides; BMI, body mass index. Data are given as mean ± SD or in median ± range.

Table 5. Toll-like receptor (*TLR*)*4* A(299)G genotype distribution and clinical characteristics in transplanted patients with (Tx15) long-term graft survival, with acute rejection (AR) and in consecutively transplanted recipients (Tx) compared with healthy controls.

TLR4 A(299)G	AA			AG			GG		
Groups	Tx	Tx15	AR	Tx	Tx15	AR	Tx	Tx15	AR
n	100	41	21	5	7	–	–	–	–
Males/females	69/31	19/22	12/9	5/0	3/4	–	–	–	–
Age (years)	53 ± 12	54 ± 12	45 ± 12	47 [22–74]	42 [32–65]	–	–	–	–
Creatinine (μmol/l)	147 ± 48	150 ± 80	645 ± 220	113 [103–168]	106 [97–181]	–	–	–	–
Proteinuria (g/day)	1.1 ± 0.4	1.5 ± 0.7	2.9 ± 2.6	1.3 [0.8–2.3]	1.2 [0.1–1.7]	–	–	–	–
Chol (mmol/l)	6.0 ± 1.2	5.8 ± 1.4	5.0 ± 1.7	5.1 [4.3–6.8]	4.9 [3.6–12.5]	–	–	–	–
TG (mmol/l)	2.5 ± 1.3	1.8 ± 0.8	2.2 ± 0.9	2.7 [1.7–4.4]	2.3 [0.8–5.1]	–	–	–	–
BMI (kg/m^2)	27.0 ± 4.6	24 ± 4.7	24 ± 0.7	24 [20–31]	23 [19–28]	–	–	–	–
Hypertension, %	35	68	80	50	85	–	–	–	–
Acute rejection, %	31	26	100	25	28	–	–	–	–

Chol, cholesterol; TG, triglycerides; BMI, body mass index. Data are given as mean ± SD or in median ± range.

itself is detrimental, as it is more frequently present in theTx and AR groups compared with healthy subjects or Tx15 patients (Table 2).

Our data indicate that healthy subjects and patients with long-term allograft survival (Tx15) share the same genetic distribution of *HSPA1B* A(1267)G, which suggests that Tx15 patients do not have increased, but 'normal, physiological' susceptibility against risk factors of transplantation in contrast to members of the more susceptible AR group.

HSP70s have been shown to stimulate the Toll and interleukin-1 receptor signaling pathways, which engage TLR2 and TLR4. We found that the *TLR4* (299)AG genetic variant, and the presence of the G allele were more frequent among patients with long-term graft survival compared with the consecutively transplanted patients, and this tendency was also observed in the AR group (Table 1). This suggests that an altered immune response in carriers of the *TLR4* (299)AG genotype might have beneficial effects on allograft survival. On the other hand, the decresed frequency of the G allele may represent a more active immune system in patients with AR.

Our hypothesis is strongly supported by the recent literature findings that the presence of the *TLR4* (299)G allele is associated with systemic inflammatory hyporesponsiveness, decreased proinflammatory cytokine and acute phase protein release [20]. It has also been reported that mice with targeted disruption of the TLR signal adaptor protein MyD88 were unable to reject incompatible allografts. Moreover, it is likely that TLRs themselves are involved in allograft rejection, as in the absence of TLRs, recipients show delayed rejection [5,21]. In lung transplant recipients *TLR4* (299)AG heterozygosity was associated with a reduced rate of acute rejections [7], which is in line with our observations in renal recipients.

From clinical data we found that serum creatinine and proteinuria were higher in the AR group compared with the others. This is not surprising as these factors are major characteristics of graft nonfunction. Hypertension occurred frequently among Tx15 patients compared with the Tx group, which is obviously a result of prolonged immunosuppressive therapy. No other difference (age, gender, cholesterol, triglycerides, etc.) was determined in the predisposing parameters among any of the study groups.

In summary, the increased frequency of *HSPA1B* (1267)GG variant with decreased HSP72 mRNA expression and altered stress protection in Tx and AR groups, indicate that decreased cytoprotective functions of HSP70s might be important in preserving graft function. This supports the assumption that during transplantation, decreased expression of HSP72 possibly influences protection against stress factors and thereby might reduce renal allograft survival. On the other hand, the presence of the TLR4 (299)G allele, which is associated with attenuated immunoreaction against the transplanted graft, might have beneficial effects against ARs and possibly improve long-term graft survival.

Our data suggest that the recipient's genetic predisposition to high (or low) HSP72 production and TLR4 activation might predict long-term allograft survival and imply that future studies of genetic polymorphisms would be helpful in elucidating the genetic risk for rejection episodes. Additional multicenter studies with a larger number of patients would be useful to investigate other factors (e.g. donor's genetic constellation, demographic and immunologic variables) which potentially influence long-term graft functions.

Acknowledgements

This work was supported by grants from the Institute for Clinical and Experimental Medicine (MZO 00023001), OTKA F042563-F048842-T37578-T046155 by the National Office for Research and Technology (NKTH), Szentágothai János Knowledge Centre, and DAAD 13-2004-2005. A.F. and A.S. are recipients of Bolyai scholarship.

We are indebted to Emílie Šimáčková, Michaela Prokopová, Mária Bernáth, and Réka Körmendy for technical assistance and Manfred Fliegauf for careful reading of the manuscript.

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