Investigation of promoter variations in dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) (CD209) and their relevance for human cytomegalovirus reactivation and disease after allogeneic stem-cell transplantation

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ABSTRACT

Promoter variations in Toll-like receptor genes (n = 7) and genes encoding pathogen recognition and virus entry receptors (n = 7) were screened to detect any association with human cytomegalovirus (hCMV) reactivation and disease in patients following allogeneic stem-cell transplantation. Two single nucleotide polymorphisms (rs735240, G>A; rs2287886, C>T) in the promoter region of the dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) showed a significant association with an increased risk of development of hCMV reactivation and disease. Furthermore, these genetic markers influenced the expression levels of DC-SIGN on immature dendritic cells, as well as the infection efficiency of immature dendritic cells by hCMV, as determined by hCMV immediate–early antigen staining. Screening of patients following allogeneic stem-cell transplantation for the presence of these defined genetic polymorphisms might help to predict the individual risk of hCMV reactivation and disease.

Keywords Allogeneic stem cell transplantation, DC-SIGN, human cytomegalovirus, promoter variations, reactivation, Toll-like receptor

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INTRODUCTION

Human cytomegalovirus (hCMV) is a ubiquitous β-herpes virus that interacts with various target cells during acute and latent infection. hCMV is one of the most significant infectious complications for allogeneic stem-cell transplantation (alloSCT) patients [1].

The ability of hCMV to recognise and infiltrate a wide range of cells, including dendritic cells (DCs), monocytes, macrophages, endothelial cells, epithelial cells, muscle cells and fibroblasts, suggests that hCMV makes use of different types of receptors. During the complex entry path, hCMV binds initially to heparan sulphate proteoglycans (HSPGs) on the host cell surface [2]. In a subsequent step, stronger connection is mediated by binding of glycoprotein B, a major compound of the viral capsid, to one of the cellular receptors. Several different pathogen recognition receptors (PRRs) and virus entry receptors of hCMV have been identified. Virus entry receptors comprise the epidermal growth factor receptor [3], integrins [4,5] and the dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) [6].

Adaptive, T-cell-mediated immune responses, as well as the innate immune system, usually control hCMV infection. In early innate
immunity, Toll-like receptors (TLRs) play a critical role by interacting with highly conserved pathogen-associated molecular patterns [7]. TLR2 has been shown to be activated by hCMV virions during the entry process, thereby triggering inflammatory cytokine production [8]. With regard to TLR4, bacterial components, as well as viral structures, have been detected [9]. TLR3 is a receptor for double-stranded RNA [10], TLR8 has been shown to recognise single-stranded RNA [11], and TLR9 is required for response to unmethylated CpG DNA [12].

Single nucleotide polymorphisms (SNPs) located in regulatory or coding regions are of special interest, as they may influence the functions of genes by altering gene expression and/or by changing the structure of the corresponding protein [13]. It has been demonstrated previously that SNPs in TLR genes are associated with susceptibility to inflammation [14]. However, SNPs in TLR2 (rs1898830, rs3804099, rs3804100) and TLR4 (rs2737191, rs5030728, rs1554973) are not associated with hCMV reactivation or disease [15]. The present study investigated the association between defined SNPs \( n = 14 \) in TLR3, TLR8, TLR9, EGFR, ITGB3, DC-SIGN and SDC2 (HSPG), and asymptomatic hCMV reactivation (DNAemia) and disease in patients after alloSCT. Three different patient groups were compared: patients with hCMV reactivation; patients with hCMV disease (as defined by Ljunghman et al. [16]); and patients without hCMV reactivation (controls).

**MATERIALS AND METHODS**

**Sample collection**

Between 1994 and 2003, whole blood samples (5–10 mL), anticoagulated with EDTA, were collected c. 30 days after alloSCT from 194 patients suffering from acute myeloid leukaemia, acute lymphatic leukaemia, myelodysplastic syndrome, non-Hodgkin’s lymphoma or severe aplastic anaemia. The combination of a seronegative donor and a seronegative recipient was excluded from the analysis. All patients were of European origin (median age 43 years; range 19–55 years; 54% males) and fulfilled the inclusion criteria described previously [15]. The study population was split into three groups: (i) 70 patients with hCMV reactivation (43% of whom had received \( >2 \) mg/kg body weight corticosteroids for immunosuppression); (ii) 59 patients with hCMV disease (81% with corticosteroid treatment); and (iii) 65 controls (66% with corticosteroid treatment). The retrospective analysis was approved by the local ethics committees and was conducted according to the principles of the Declaration of Helsinki.

DNA was extracted using standard methods based on spin-column technologies and was frozen at \(-80^\circ\text{C}\) until further analysis. The defined SNPs rs5743318 (TLR3, 4q35), rs2407992, rs3747414, rs5764880, rs5744077 (TLR8, Xp22), rs352140 and rs5743842 (TLR9, 3p21.3) were analysed for 69 patients with hCMV reactivation, 26 patients with hCMV disease, and 51 patients without hCMV reactivation because of limited genomic DNA yield. The association tests for the markers rs2287886 and rs735240 of DC-SIGN were based on 40 samples in each group.

**SNP analysis**

With the exception of the two SNPs in DC-SIGN, specific oligonucleotide probes for each genetic marker were designed (TIB MOLBIOL, Berlin, Germany) (Table S1, see Supplementary material). SNP analysis was performed using the LightCycler system and the LightCycler FastStart DNA Master Hybridisation Probes Kit (Roche, Mannheim, Germany). Melting curve analysis was performed using a temperature transition rate of 0.1°C/s.

Analysis of the SNPs rs2287886 and rs735240 (DC-SIGN) was performed by matrix-assisted laser desorption ionisation mass spectrometry (MALDI-MS) (Autoflex; Bruker Daltonics, Bremen, Germany). PCR products were treated with shrimp alkaline phosphatase, followed by extension of SNP-specific biotinylated primers containing a photo cleavable linker (BioTez, Berlin, Germany) using TERRpol polymerase (Solis BioDyne, Tartu, Estonia). Products were purified using the Genostrep 96 Kit (Bruker Daltonics), the primers were cleaved using UV light, and the mass of the extended oligonucleotides was determined by MALDI–time of flight (TOF) analysis.

**Generation of DCs and DC-SIGN expression analysis**

Culture of immature DCs (iDCs) used 100 mL of blood from five healthy individuals with either the CA or TG haplotype across markers rs735240 and rs735240. Each individual was homozygous for the C, respectively T, allele of marker rs735240, and the A, respectively G, allele of marker rs2287886. Isolation of monocytes and differentiation into iDCs was achieved by stimulation with interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor as described previously [17]. After 6 days, the cells were harvested and the expression of DC-SIGN was analysed by real-time PCR subsequent to isolation of total RNA and reverse transcription [18]. The primers and probes used were: DC-SIGN F, 5'-CTGAGGAGCGAAGCTTCTCCTACA; DC-SIGN R, 5'-GCAAGATTACATTGGTCGCTG; DC-SIGN FL, 5'-TGTGGCCACGCTTCAAGCAGTA; and DC-SIGN LC, 5'-GGAACAGGAGGGCCCAAC.

**hCMV infection and immunocytochemistry**

iDCs were infected with hCMV strain TB40E [17] and stained for immediate–early (IE) antigens as described previously [19] with minor modifications. In brief, the cells were resuspended in medium containing IL-4 and granulocyte-macrophage colony-stimulating factor, and then incubated with hCMV suspension or fresh medium for 2 h at 37°C. The cells were then rinsed and cultured in 240-well plates at a density of 1–1.5 \( \times 10^6 \) cells/mL. For determination of infection efficiency, iDCs were collected 24 h after infection, cytoscintrifuged on to glass slides, and fixed with acetone. hCMV IE antigen
expression was detected by indirect immunofluorescence staining. The slides were incubated with monoclonal antibody E13 directed against the non-structural IE proteins IE72 and IE86 (pUL122/123; Biosoft, Paris, France) in an appropriate dilution for 1 h at 37°C, and then with Cy3-conjugated goat anti-mouse Ig-Fab2 polyclonal serum (Jackson Immuno Research, West Grove, PA, USA) in an appropriate dilution under the same conditions. Nuclei were counterstained with 4,6-diamidino-2-phenylindole.

Statistical analysis

Statistical analysis was performed as for a classic case-control study using the allele-frequency-difference test to test for single-marker associations. As TLR8 is localised on the X-chromosome, allele frequencies were determined separately for each gender. The level of significance was set to $p < 0.01$ [20,21]. In order to detect population admixture or genotyping artefacts, each marker was tested for Hardy–Weinberg equilibrium in both the case and the control group. To analyse the impact of the genetic components and clinical risk-factors on hMCV status, a logistic regression analysis was performed using SAS v.9.1 (SAS Inc., Cary, NC, USA). The patient groups with hCMV reactivation and disease were treated as categorical variables, using the controls as the reference category for the outcome, whereas the genotypes of the markers rs2287886 and rs735240 and the clinical risk-factors (graft vs. host reaction, CD34 selection, corticosteroid treatment >2 mg/kg body weight) were treated as explanatory variables. A reduced model was tested, taking only the main effects into account, and the correlation matrix of the model was derived to highlight the relationship between the genetic and clinical factors.

RESULTS

Following alloSCT, the patient call rate (i.e., the percentage of DNA samples that could be analysed) using the LightCycler assay (97.3%) and MALDI-MS (93.3%) were within tolerable ranges. No significant association ($p < 0.01$) between genetic markers in TLR8, TLR9, EGFR, ITGB3 and SDC2 and hCMV reactivation or disease was revealed (Table 1). Four markers were revealed to be monomorphic, i.e., TLR3 (rs5743318), TLR8 (rs5744077), TLR9 (rs5743842) and ITGB3 (rs5921). However, if the level of significance was set to $p < 0.05$, association analysis for TLR8 and SDC2 revealed that the frequencies of three defined alleles were increased in patients with hCMV reactivation/disease. Thus, rs3764880 (TLR8) was associated with hCMV reactivation ($p = 0.050$), and rs3747414 (TLR8) and rs1042381 (SDC2) were associated with hCMV disease ($p = 0.039$ and $p = 0.037$, respectively).

Table 1. Association between genetic polymorphisms in TLR8, TLR9, DC-SIGN, EGFR, ITGB3 and SDC2 and human cytomegalovirus (hCMV) reactivation and disease

<table>
<thead>
<tr>
<th>Gene</th>
<th>dbSNP number</th>
<th>Nucleotide position</th>
<th>Allele</th>
<th>Control</th>
<th>Reactivation</th>
<th>Disease</th>
<th>p values</th>
<th>Control vs. Reactivation</th>
<th>Control vs. Disease</th>
<th>Reactivation vs. Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR8 (Xp22)</td>
<td>rs2407992</td>
<td>10 608 C/G</td>
<td>C/C</td>
<td>11</td>
<td>18</td>
<td>6</td>
<td>$\geq 0.912$</td>
<td>$\geq 0.654$</td>
<td>$\geq 0.394$</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C/G</td>
<td>11</td>
<td>9</td>
<td>4</td>
<td>$\geq 0.688$</td>
<td>$\geq 0.425$</td>
<td>$\geq 0.174$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>G/G</td>
<td>26</td>
<td>39</td>
<td>16</td>
<td>$\geq 0.474$</td>
<td>$\geq 0.473$</td>
<td>$\geq 0.719$</td>
<td></td>
</tr>
<tr>
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<td></td>
<td>10 908 A/C</td>
<td>A/A</td>
<td>9</td>
<td>17</td>
<td>3</td>
<td>$\geq 0.090$</td>
<td>$\geq 0.068$</td>
<td>$\geq 0.039$</td>
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<td>rs3764880</td>
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<td>−3679 A/G</td>
<td>A/A</td>
<td>38</td>
<td>48</td>
<td>19</td>
<td>$\geq 0.523$</td>
<td>$\geq 0.884$</td>
<td>$\geq 0.811$</td>
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<td>TLR9 (3p21.3)</td>
<td>rs352140</td>
<td>1464 C/T</td>
<td>C/C</td>
<td>12</td>
<td>24</td>
<td>4</td>
<td>$\geq 0.515$</td>
<td>$\geq 0.411$</td>
<td>$\geq 0.068$</td>
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<td></td>
<td>C/T</td>
<td>24</td>
<td>18</td>
<td>12</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>T/T</td>
<td>15</td>
<td>17</td>
<td>9</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>DC-SIGN (19p13)</td>
<td>rs2267886</td>
<td>−139 C/T</td>
<td>C/C</td>
<td>17</td>
<td>11</td>
<td>24</td>
<td>0.197</td>
<td>0.087</td>
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<td>12</td>
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<td></td>
<td></td>
<td></td>
<td>T/T</td>
<td>4</td>
<td>6</td>
<td>2</td>
<td></td>
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<tr>
<td>rs75240</td>
<td></td>
<td>−939 A/G</td>
<td>A/A</td>
<td>13</td>
<td>3</td>
<td>8</td>
<td>$\geq 0.010$</td>
<td>$\geq 0.034$</td>
<td>$\geq 0.598$</td>
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<td></td>
<td></td>
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<td>A/G</td>
<td>16</td>
<td>19</td>
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<td></td>
<td></td>
<td>G/G</td>
<td>7</td>
<td>12</td>
<td>15</td>
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<tr>
<td>EGFR (7p21)</td>
<td>rs1543848</td>
<td>142 285 A/G</td>
<td>A/A</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>0.757</td>
<td>0.690</td>
<td>0.918</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>A/G</td>
<td>17</td>
<td>18</td>
<td>19</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>G/G</td>
<td>35</td>
<td>39</td>
<td>32</td>
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<tr>
<td>rs10251977</td>
<td></td>
<td>162 093 A/G</td>
<td>A/A</td>
<td>26</td>
<td>18</td>
<td>23</td>
<td>0.966</td>
<td>0.974</td>
<td>0.971</td>
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<td></td>
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<td>A/G</td>
<td>22</td>
<td>28</td>
<td>19</td>
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<tr>
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<td></td>
<td></td>
<td>G/G</td>
<td>11</td>
<td>5</td>
<td>10</td>
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<td></td>
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<td></td>
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<tr>
<td>ITGB3 (17q21.32)</td>
<td>rs1270949</td>
<td>37 110 A/C</td>
<td>A/A</td>
<td>27</td>
<td>21</td>
<td>25</td>
<td>0.066</td>
<td>0.892</td>
<td>0.055</td>
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<td></td>
<td>A/C</td>
<td>28</td>
<td>27</td>
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<td></td>
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<td></td>
<td>C/C</td>
<td>7</td>
<td>15</td>
<td>6</td>
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<tr>
<td>SDC2 (8q22–q23)</td>
<td>rs1042381</td>
<td>108 162 A/T</td>
<td>A/A</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.304</td>
<td>0.274</td>
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<td>21</td>
<td>15</td>
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<td></td>
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<td></td>
<td>T/T</td>
<td>43</td>
<td>48</td>
<td>33</td>
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<td></td>
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</tr>
</tbody>
</table>

1Positions of the single nucleotide polymorphisms (SNPs) in the respective genes were determined at http://snpper.chip.org.
2Patients in the control group did not show hCMV reactivation. Numbers of homozygous and heterozygous patients used for statistical comparison of the respective patient groups are specified.
3As TLR8 is localised on the X-chromosome, allele frequencies were determined separately according to the gender of the donor.
In addition, two SNPs localised in the promoter region of DC-SIGN (rs735240 and rs2287886) were revealed to be associated significantly with hCMV reactivation or disease. Thus, the G allele of rs735240 was associated with hCMV reactivation when compared with the control group (p = 0.01, OR = 2.41, 1.22–4.75), and the C allele of rs2287886 was found more frequently in the hCMV disease group than in the hCMV reactivation group (p = 0.003, OR = 2.88, 1.41–5.88). Furthermore, both markers were associated with hCMV disease when compared with the control group (p = 0.034, OR = 2.01, 1.05–3.86; and p = 0.087, OR = 1.88, 0.91–3.87, respectively).

In order to determine whether healthy individuals, who have been identified previously to be primarily homozygous for CC or TT (rs735240) and AA or GG (rs2287886), had different levels of expression of DC-SIGN, expression rates were analysed on iDCs. It has been demonstrated previously that acquisition of DC-SIGN is induced primarily by IL-4 during the monocyte–DC differentiation pathway [22]. Depending on the corresponding genotype, iDCs from individuals with the CA haplotype expressed significantly higher levels of DC-SIGN in comparison with individuals with the TG haplotype (Fig. 1; p = 0.04).

Experiments with IE-immunofluorescence staining showed that iDCs with enhanced expression of DC-SIGN (CA haplotype) were more susceptible to infection with the clinically important hCMV strain TB40E (29.6 ± 13.9%) than iDCs generated from individuals with the TG haplotype (8.6 ± 5.5%; p = 0.037).

By analysing the clinical risk-factors, it was revealed that acute graft vs. host disease grade II–IV was associated significantly with hCMV disease (p = 0.042). In addition, patients treated with corticosteroids (>2 mg/kg body weight) developed hCMV disease significantly more frequently than patients treated with a lower dose of steroid (p = 0.023). Logistic regression analysis confirmed the significant impact of the genetic markers on the hCMV group classification, whereas the effects of the clinical risk-factors were negligible. The logistic regression model revealed no high correlations between markers and a clinical risk-factor (Table S2, see Supplementary material).

**DISCUSSION**

This study analysed genetic polymorphisms in TLR genes and genes encoding PRRs for their potential association with hCMV reactivation and disease. It is well-known that different viral pathogens, including hCMV, interact with TLRs [7]. However, most previous studies have focused on an analysis of associations between genetic markers in TLR2 and TLR4 and bacterial infections [14], and only limited data exist concerning the association of genetic markers in TLR genes with the occurrence of virus infections. A polymorphism (rs5743708, Arg753Gln) has been identified in TLR2 that is associated with an increased risk of hCMV disease after liver transplantation [23]. TLR3 and TLR9 have been reported to trigger the innate immune defence mechanisms against mouse CMV infection, and TLR9 also appears to be involved in immune defence against herpes simplex virus [24,25]. However, the present study failed to identify any markers in TLR3 and TLR9 that contributed to hCMV susceptibility.

To our knowledge, no data exist concerning an interaction between TLR8 and hCMV, and it is not known whether the association of rs3764880 with hCMV reactivation is coincidental or indicates a possible role of TLR8 in the innate immune response to hCMV. Analysis of PRR genes has revealed an association between the X allele of rs1042381 (SDC2) and the occurrence of hCMV disease (as compared to patients with hCMV reactivation only; p = 0.037). As mentioned above, hCMV interacts with HSPGs during the early
stages of infection. Further analysis should examine whether the amino-acid substitution from serine to threonine has an effect on the susceptibility of host cells to hCMV. The function of DC-SIGN as a PRR is not fully understood, and this is a controversial topic [26–28]. DC-SIGN (CD209) facilitates binding and internalisation of several viruses (e.g., human immunodeficiency virus-1), bacteria (e.g., *Mycobacterium tuberculosis*) and parasites (e.g., *Leishmania* and *Schistosoma*) on DCs. During the development of human monocyte-derived DCs, DC-SIGN becomes organised in well-defined micro-domains on the plasma membrane. This organisation seems to be important for binding and internalisation of virus particles, suggesting that these multi-molecular assemblies of DC-SIGN act as a docking site for pathogens to invade the host [27]. In contrast, Geijtenbeek et al. [28] reported that DC-SIGN does not function as a receptor for viral entry into DCs, but instead promotes efficient infection in trans of cells that express CD4 and chemokine receptors.

The present analysis identified two genetic markers with significant associations: (i) rs735240, which is associated with hCMV reactivation; and (ii) rs2287886, whose allelic pattern differed significantly between hCMV reactivation and disease. These results were in agreement with previous studies of the same gene region. Barreiro et al. [29] described two promoter variations of DC-SIGN, at positions −871 (rs735239) and −336 (rs4804803), that were associated with tuberculosis in a South African cohort. Both polymorphisms can also be found with a frequent distribution in a Caucasian population, and are localised next to rs735240 and rs2287886. In addition, Sakuntabhai et al. [30] showed that the −336G allele of rs4804803 was associated with strong protection against dengue fever, as opposed to dengue haemorrhagic fever. This study concluded that the −336A (rs4804803) allele increases the expression level of DC-SIGN, thereby probably leading to better capture of pathogens and enhanced induction of the adaptive immune response. Furthermore, Martin et al. [31] found that Americans of European origin who were at risk for parenteral human immunodeficiency virus infection were more likely to carry the −336C SNP than the −336T SNP.

Halary et al. [6] demonstrated that hCMV can be captured by DCs via binding on DC-SIGN, and that hCMV envelope glycoprotein B is a viral ligand for DC-SIGN. The present study revealed that different DS-SIGN alleles of rs735239 and rs4804803 influenced the expression levels of DC-SIGN. Individuals who were homozygous for rs735240 (CC) and rs2287886 (AA) showed significantly higher DC-SIGN expression levels than individuals with TT/GG genotypes (p 0.04). Furthermore, a significantly higher efficiency of infection for DCs with the CA haplotype was shown than for DCs with the TG haplotype (p 0.037). To our knowledge, this is the first report of an association between defined polymorphisms in DC-SIGN and the amount of intracellular hCMV IE antigen. These findings provide strong evidence of a functional correlation between the defined alleles in DC-SIGN and hCMV infection efficacy. These results agree with those of Flint et al. [32], who suggested that polymorphisms in CD81 (which is a host-cell receptor for hepatitis C virus) may contribute to hepatitis C virus susceptibility.

It is unlikely that nucleotide exchanges are the only cause of hCMV reactivation. Other risk-factors may also have a causal function. The higher risk of hCMV reactivation for serologically positive recipients is undisputed [33], whereas the influence of the donor’s serological status on the outcome is still unclear [34]. In the present analysis, no association was revealed between the hCMV status of the donor and hCMV reactivation and disease. However, acute graft vs. host disease (p 0.042), CD34 selection (p 0.037) and treatment with corticosteroids (p 0.023) were, as expected, associated with hCMV disease.

In conclusion, screening of patients for defined polymorphisms following alloSCT helps to predict the individual risk of the development of hCMV reactivation and disease. These genetic polymorphisms may also influence the development of DC-SIGN-based prophylaxis and therapy in terms of efficiency and safety.

**Acknowledgements**

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technological excellence in research and therapy of leukemia (CML, AML, ALL, CLL, MDS, CMF) by integration of the leading national leukemia networks and their interdisciplinary partner groups in Europe, project no. 503216. We thank O. Morton for correction of the English language. The authors declare that they have no conflicts of interest in relation to this work.

SUPPLEMENTARY MATERIAL

The following Supplementary material for this article is available online at http://www.blackwell-synergy.com:

Table S1. Sequences of primers and probes used for single nucleotide polymorphism genotyping

Table S2. Regression analysis of the significant genetic markers and the clinical risk-factors analysed

REFERENCES