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Short communication

The resistance mutation R155K in the NS3/4A protease of hepatitis C virus also leads the virus to escape from HLA-A*68-restricted CD8 T cells

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ABSTRACT

The NS3/4A serine protease of the hepatitis C virus (HCV) is one of the most attractive targets for specific antiviral agents. However, mutations conferring resistance may decrease the efficacy of these drugs. Although the level of resistance associated with specific mutations differs between different compounds, substitutions R155K and A156T reduce susceptibility to all protease inhibitors published so far. Interestingly, variants harboring the resistant mutation R155K were also detected as the predominant quasispecies in some treatment-naïve patients. Of note, key positions for resistance overlap with the HLA-A*68-restricted epitope $HAVGIFRAAV_{1175–1184}$. The aim of our study was to analyze the impact of protease inhibitor resistance mutations on the replication level and the antiviral CD8 T cell response against this HCV epitope. Our findings suggest that the R155K variant is associated with a relatively high replication level and with a substantial loss of cross-recognition by specific CD8 T cells targeting the epitope $HAVGIFRAAV_{1175–1184}$, providing a possible explanation for its existence in the absence of drug selection pressure.

The NS3/4A serine protease of the hepatitis C virus (HCV) is one of the most attractive targets for specific antiviral agents. Several protease inhibitors (PIs) are currently in preclinical and clinical development and demonstrate enormous potential for suppression of viral replication both *in vitro* and *in vivo* (Thompson et al., 2009). However, mutations conferring resistance may decrease the efficacy of these drugs. Recent studies have demonstrated the rapid selection of drug resistance mutations to novel PIs *in vitro* and *in vivo* (Koev and Kati, 2008; Sarrazin et al., 2007; Tong et al., 2008). Mutations in the binding region for PIs seem to be the key substitutions for resistance to this class (Koev and Kati, 2008). Although the level of resistance associated with specific mutations differs between different compounds, substitutions R155K and A156T reduce susceptibility to all PIs published so far (He et al., 2008; Kim and Timm, 2008; Koev and Kati, 2008). The frequency of resistance mutations in the protease domain of NS3 in treatment-naïve patients was recently analyzed in three different large cohorts (Bartels et al., 2008; Gaudieri et al., 2009; Kuntzen et al., 2008). Although predominant mutations at position A156 were absent from all isolates included in these studies (total of 906 genotype 1a and 419 genotype 1b protease sequences), variants harboring the resistant mutation R155K were detected as the predominant quasispecies in seven treatment-naïve patients infected with HCV genotype 1a. The appearance of this variant *in vivo* in the absence of a PI suggests either relatively low fitness costs at least in some isolates or selection by other forces than drug pressure.

The antiviral immune response by CD8 T cells is believed to play an important role in containment of acute HCV infection. One of the mechanisms for T cell failure and consequently viral persistence is mutational escape from immunodominant CD8 T cell responses during the early phase of infection. Indeed, selection pressure stimulated by the adaptive immune response is an important driving force for the evolution of HCV. The immunodominant epitope presented by HCV-infected patients carrying the HLA-A*68-allele is located in the protease domain of HCV NS3 ($HAVGIFRAAV_{1175–1184}$). So far, a T cell response to this epitope has only been described in subjects with spontaneous control of HCV infection. Interestingly, the key positions R155 and A156 conferring resistance against a broad range of PIs overlap with this epitope. Therefore, immune pressure prompted by CD8 T cells targeting this epitope may select the R155K variant in some patients. The aim of our study was to analyze the impact of the described PI resistance mutations overlapping with the HLA-A*68-restricted epitope $HAVGIFRAAV_{1175–1184}$ on the replication level and the antiviral CD8 T cell response against this HCV epitope.

A transient replication assay based on the subgenomic HCV replicon con1 (pFK PI-luc/NS3-3′/Con1/ET kindly provided by Ralf

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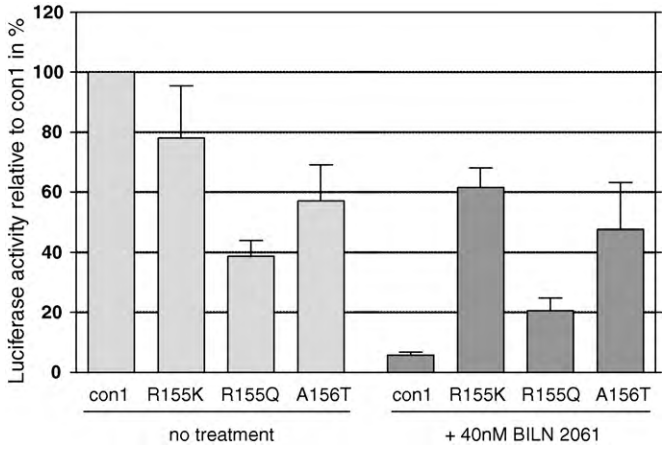

Fig. 1. Relative replication efficiency of different variants and the impact of the protease inhibitor BILN 2061. Luciferase activities after 48 h are shown relative to the con1 prototype in the absence of BILN 2061 (set to 100%). Error bars represent standard deviations of three independent experiments.

Bartenschlager) that encodes the firefly luciferase as a reporter gene was used (Lohmann et al., 2003). In this backbone, mutations encoding the variant R155K, R155Q and A156T were introduced by site-directed mutagenesis and confirmed by sequencing as previously described (Salloum et al., 2008). R155Q was tested because it was described as one of the primary resistance mutations that was selected in the presence of the protease inhibitor BILN 2061, although it has not been observed *in vivo* up to now. The plasmid variants were then linearized and served as a template for RNA synthesis. *In vitro* transcribed RNA was transfected into Huh7 cells and luciferase activity was determined after 48 h in the absence or presence of BILN 2061 (kindly provided by Boehringer Ingelheim). The results of the luciferase activity are shown as percent relative to the luciferase activity of con1 in the absence of protease inhibitor treatment (Fig. 1). Replication of the R155K variant is only modestly impaired compared to con1 (78%), suggesting relatively low fitness costs as previously shown (Zhou et al., 2007). The R155Q variant has the highest fitness costs. This is in line with the appearance of the R155K variant *in vivo*, even in the absence of drug selection pressure (Colson et al., 2008; Kim et al., 2009) and the complete absence of the R155Q *in vivo* (Bartels et al., 2008; Gaudieri et al., 2009; Kuntzen et al., 2008). The A156T variant has an intermediate replication level of 57% compared to con1 and, so far, has only been described as a low frequency variant isolated from the intrahepatic compartment of a patient with chronic HCV infection (Cubero et al., 2008). The intermediate replication level of the A156T variant in this study differs from some of the previously reported results. In the N-strain genotype 1b replicon a replication level of less than 10% compared to wild type was reported (Lu et al., 2004; Yi et al., 2006). However, in a colony formation assay with the same replicon that was used in our study (con1/ET) McCown et al. (2008) reported a replication efficiency of 50% highlighting that the replication capacity of such variants also depends on the genetic background of the employed replicon. The relevance of such differences in the replication level determined *in vitro* for the situation *in vivo* is still unclear. Of note, the R155K variant was relatively stable for months in some patients, thus supporting the hypothesis that differences in the replication level as observed here *in vitro* are relevant also *in vivo* (Sarrazin et al., 2007). In contrast, the A156T variant rapidly reverted back to prototype within a few days *in vivo* when treatment with a PI was discontinued (Sarrazin et al., 2007). Along this line, an escape mutation in an HLA-B*08-restricted CD8 T cell epitope that rapidly reverted in the absence of immune pressure has a similar intermediate replication level of 60% compared to wild type (Salloum et al., 2008), supporting that minor differences in the replication rate may be important *in vivo* in the presence of a high viral turnover. As previously shown, in the presence of 40 nM BILN 2061 (10 times IC_{50}) the replication level of con1 is decreased to 6%. In the presence of such high concentrations of the drug the R155K shows the highest replication level followed by the A156T and the R155Q variant, supporting their role in resistance against this compound in line with previous results (He et al., 2008; Tong et al., 2008; Zhou et al., 2007).

Previously a genotype 1a HCV-infected patient with dynamic fluctuations in the quasispecies composition was reported with alternating predominance of prototype or the R155K substitution (Kim et al., 2009). In this subject no T cell associated immune pressure as the driving force for this continuous evolution was detected. It remains unclear whether the R155K, R155Q or the A156T substitution has an impact on the T cell response directed against the overlapping HLA-A*68-restricted CD8 epitope HAVGIFRAAV. To address this issue, T cells were expanded from cryopreserved PBMCs from an HLA-A*68-positive subject who spontaneously resolved HCV infection (anti-HCV positive and HCV RNA negative) as previously described (Giugliano et al., 2009). After 10 days the cells were restimulated with the same peptide or with the R155K, R155Q and A156T variants and the number of interferon gamma-producing cells was determined by intracellular cytokine staining (Fig. 2). Upon restimulation with the prototype peptide 8.34% of all CD8 T cells secreted interferon gamma. Although the A156T variant was cross-recognized to a similar extent (6.47%), the number of IFNg+/CD8+ T cells was substantially decreased upon restimulation with the R155K variant (2.25%) and almost absent upon restimulation with the R155Q variant (0.40%). Importantly, we were also unable to expand specific T cells upon stimulation with the R155K or the R155Q variant peptides (data not shown). These variants therefore enabled immune escape from HLA-A*68-restricted CD8 T cells in this patient. Of note, the originally mapped epitope sequence differed in one amino acid position compared to our current sequence (Lauer et al., 2004). The reference genotype 1a sequence H77 as the basis for the original epitope sequence harbors an I153L substitution, however, this substitution is overall rare in genotype 1a and 1b. About 98.7% of the genotype 1a sequences and 96.9% of the genotype 1b isolates in the HCV sequence database carry isoleucine in position 153. We therefore believe that the impact of the R155K substitution as described in the current study would play a role in T cell recognition of the majority of genotype 1a and 1b isolates.

Taken together, the R155K mutation is relatively fit *in vitro* and, possibly, *in vivo*, reduces susceptibility to treatment with PIs and substantially decreases recognition by HLA-A*68-restricted CD8 T cells. However, it is unclear if there is a preferential selection of this mutation in HLA-A*68-positive patients. The epitope region is overall highly conserved in genotype 1 when sequences from the public database are analyzed. The most frequent polymorphisms in the epitope region are in position 2 (A to V) and in position 3 (V to A) in genotype 1a and 1b. Of note, these polymorphisms represent the consensus sequence in genotype 3 and are fully cross-reactive with specific CD8 T cells against this epitope. HLA-A*68 is a relatively frequent allele in Caucasian populations with reported phenotype frequencies between 5% and 15% (www.allelefrequencies.net). If the R155K variant is the result of immune selection pressure, it clearly must be a rare event. A study by Gaudieri et al. (2009) specifically addressed this question. However, the low frequency of the R155K variant in treatment-naïve subjects may have precluded identification of HLA-A*68-associated selection pressure. We similarly sequenced the protease domain from 15 HLA-A*68-positive patients infected with genotype 1 (nine genotype 1b and six genotype 1a) and did not observe the R155K substitution (data not shown). An association between this allele and the presence of

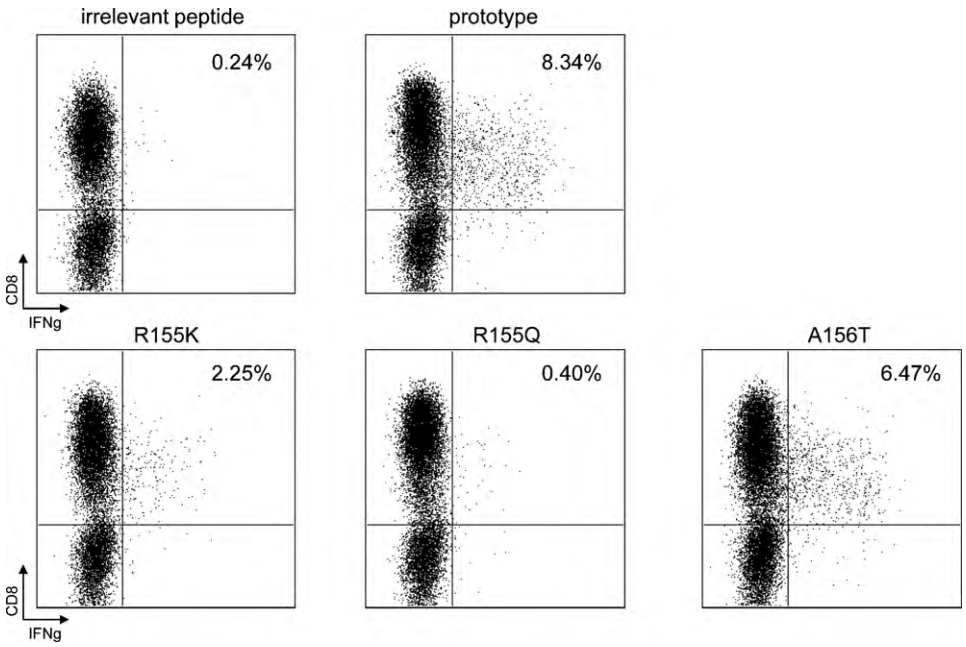

Fig. 2. Cross-recognition of T cells specific for the HLA-A*68-restricted epitope HAVGIFRAAV. T cells were expanded from cryopreserved PBMCs for 10 days in the presence of the prototype peptide (1 μg/ml) and then restimulated for 5 h with different variants of the peptide (10 μg/ml) as indicated. The number of IFNg+/CD8+ T cells is indicated in the upper right quadrant of each dot plot (relative to all CD8+ T cells in percent). An irrelevant peptide served as a negative control.

the R155K mutation seems therefore unlikely, although it cannot be fully excluded.

What are the consequences of the observed overlap between resistance and immune escape associated with the R155K variant when sequence data suggest that the R155K variant is overall rarely selected in treatment-naïve subjects, even in the presence of the relevant HLA-A*68-allele? Current data suggest that R155K is a mutation that is rapidly selected in patients treated with protease inhibitors including telaprevir and boceprevir which are both currently in phase III trials and are expected to be available soon. The frequency of the R155K variant in circulating isolates will then depend on the success of treatment with this new class of antiviral agents. Each treatment failure likely increases the number of circulating isolates harboring resistance mutations and the relatively fit and stable R155K variant is potentially particularly problematic. In an HLA-A*68-positive subject with chronic HCV infection, drug selection of the "immune escape" variant will probably be without any consequences as viral persistence was already established irrespective of the ongoing immune response. However, although blood supplies are essentially safe nowadays, transmission of HCV still frequently occurs in high risk groups such as injection drug users (Esteban et al., 2008). Transmission of a resistant virus with the R155K mutation to an HLA-A*68-positive individual will potentially impair the immune response. This epitope seems to be one of the immunodominant targets in HLA-A*68-positive subjects and has so far only been detected in subjects after a spontaneous resolution of HCV infection (Giugliano et al., 2009; Lauer et al., 2004). Impairment of such a response could therefore have important consequences for the ability to achieve immune control. Clearly, the new era of protease inhibitors as treatment options for HCV will be welcomed. Widespread use of these drugs will change the frequency of polymorphisms in circulating isolates with possible downstream effects on future treatment outcome and immune responses directed against HCV.

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