Combined Administration of RG7652, a Recombinant Human Monoclonal Antibody Against PCSK9, and Atorvastatin Does Not Result in Reduction of Immune Function

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RG7652 is a human IgG1 monoclonal antibody designed to inhibit proprotein convertase subtilisin/kexin type 9 (PCSK9) binding to hepatic low density lipoprotein receptor (LDL-r), thereby blocking PCSK9-mediated degradation of LDL-r. This therapeutic candidate is under development for the prevention of cardiovascular mortality and morbidity in dyslipidemic patients. The primary objective of this study was to evaluate the potential immunotoxicological effects of RG7652 when given to cynomolgus monkeys either alone or in combination with a daily oral dose of atorvastatin. Administration of RG7652 via subcutaneous injection every other week for 12 weeks (a total of seven doses), daily oral doses of atorvastatin (total of 85 doses), and combinations of each up to 15 and 20 mg/kg/dose, respectively, were well tolerated and there was no evidence of alteration in immune function. Administration of pharmacologically relevant doses of RG7652 in combination with atorvastatin to healthy monkeys does not result in clinically meaningful immunosuppression as measured by T-cell dependent antibody responses, natural killer cell activity, immunophenotype, or delayed type hypersensitivity. The only pharmacologically mediated changes observed during the dosing period were the anticipated changes in circulating cholesterol.

Key Words: PCSK9; therapeutic monoclonal antibody; nonhuman primate; atorvastatin; immunotoxicology.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a serine endoproteinase enzyme that binds to the epidermal growth factor-like repeat A (EGF-A) domain of cell surface LDL-receptors (LDL-r) and facilitates lysosomal destruction of LDL-r upon receptor internalization (Kwon et al., 2008; Maxwell and Breslow, 2005; Zhang et al., 2007). RG7652 is an anti-PCSK9 human IgG1 specifically designed to block PCSK9 binding to the hepatic LDL-r, thereby increasing cell surface density of LDL-r and decreasing circulating LDL-cholesterol (LDL-c). RG7652 is currently being developed for the prevention of cardiovascular (CV) mortality and morbidity when added to standard of care in dyslipidemic patients with elevated LDL-c despite optimized pharmaceutical intervention and lifestyle changes. Statins (HMG-CoA reductase inhibitors) are currently the most widely prescribed drug class for treatment of hypercholesterolemia, and a combination of RG7652 and atorvastatin has been shown to have significantly greater LDL-c lowering potential than atorvastatin alone (Tingley et al., 2013).

For therapeutics designed to lower serum cholesterol, an important consideration for both safety and efficacy is the potential to impact immune function. In addition to lipid lowering, statins are known to possess anti-inflammatory properties, which are thought to positively influence efficacy given the role inflammation plays in coronary and vascular disease (Antonopoulos et al., 2012; Chow, 2009; De Loecker and Preiser, 2012; Greenwood et al., 2006; Kuipers et al., 2005; Lingwood and Simons, 2010; Surls et al., 2012). Much of the immunomodulatory effects of statins are thought to be the byproduct of altered intracellular sterol metabolism (e.g., defective prenylation of GTPases), but direct reduction in circulating cholesterol has also been hypothesized to impact immune cell function and inflammation (Chyu et al., 2014; Kinlay, 2007; Puato et al., 2010). Plasma membrane cholesterol, particularly in lipid raft microdomains, is critical to immunological synapse formation and T-cell signaling. Modulation of membrane cholesterol in lymphocytes has been shown to impact T cell inflammatory responses (Lingwood and Simons, 2010; Surls et al., 2012) and disruption of lipid raft microdomains by statins has been proposed as an important mechanism of immunomodulation for this class of drugs (Kuipers et al., 2005). However, over the last several decades, statins have been studied in numerous randomized controlled trials and have proven to be not only effective but safe in a variety of patient populations; importantly, adverse safety events related to immunomodulation, including increased risk of cancer, have not emerged as significant hazards (Armitage, 2007; Law and Rudnicka, 2006; Mills et al., 2011; Naci et al., 2013).
Although extensively evaluated for statins, far less is known regarding the impact of PCSK9 inhibitors on immune function, either alone or in combination with statins. To address the hypothetical safety issues for this class of drugs (monoclonal antibody inhibitors to PCSK9), the FDA has specifically recommended the conduct of preclinical studies to evaluate the immunotoxicological potential of PCSK9 inhibitors in combination with statins. To support clinical development of RG7652, a preclinical combination immunotoxicology study was conducted in cynomolgus monkeys with both RG7652 and atorvastatin. The study was designed to evaluate the potential for immunotoxicological effects of RG7652 when administered to monkeys subcutaneously (SC) every other week for 12 weeks, either alone or in combination with atorvastatin administered daily by oral gavage. T cell-dependent antibody responses (TDAR) were measured by vaccine-specific IgG and IgM titers following primary and booster vaccinations with keyhole limpet hemocyanin (KLH). Delayed type hypersensitivity (DTH) responses were evaluated following vaccination with Bacillus Calmette-Guérin (BCG). Natural killer (NK) cell activity was evaluated using an ex vivo fluorescence assay and immunophenotyping of whole blood was conducted using markers for CD3, CD4, CD8, CD16, and CD20.

Finally, as a confirmation of pharmacologic activity, the impact of RG7652, alone or in combination with atorvastatin, was evaluated for LDL-, HDL-, and total-cholesterol. Although the effects of RG7652 have been studied previously in monkeys, this study provided comparative pharmacologic and toxicologic data for RG7652 when combined with statins, which modulate cholesterol levels by an independent mechanism.

MATERIALS AND METHODS

Pharmaceutical agents. RG7652 (Genentech, South San Francisco) is a recombinant antibody based on a human IgG1 framework containing heavy chain VHIII and light chain VkI subgroup sequences and is directed specifically against PCSK9 at the same site that interacts with the EGF-A domain of the LDL-r. RG7652 was provided as an aqueous solution and stored at 4–5°C prior to administration.

Atorvastatin (NDC: 60505-2671-09, Apotex Corp., Weston, FL) was received in tablet form, pulverized and formulated as a suspension in 0.5% methylcellulose (400 cps) and 0.1% tween 80 and after preparation stored under refrigerated conditions.

Animals. Male and female Chinese cynomolgus monkeys were obtained from Charles River Laboratories. Monkeys were 2–4 years of age for males (2.5–3.1 kg) and 2–5 years of age for females (2.5–2.9 kg). They were housed in the Charles River Laboratories testing facility (Reno, NV) and allowed to acclimate to the laboratory environment for a minimum of 14 days prior to initiation of treatment. Animals were housed in stainless steel cages equipped with a stainless steel mesh floor. The animal rooms were environmentally controlled to maintain a temperature of 18–29°C, a relative humidity of 30–70%, and a 12-h light/dark cycle. Certified primate diet (Purina, Catalog no. 5048) was provided daily in amounts appropriate for the size and age of animals and supplemented with fruit or vegetables 2–3 times weekly. Municipality tap water (Reno, NV) was processed through a reverse osmosis filter and passed through UV light treatment and was supplied to animals ad libitum via automatic watering device. Unless precluded for technical or scientific reasons, animals were provided with enrichment devices and food treats. Animals of the same sex were commingled within dose groups after initial compatibility tests were completed. Veterinary care was available throughout the course of the study and animals were examined by the veterinary staff as warranted by clinical signs or other changes. All study procedures were conducted according to a written study protocol and facility standard operating procedures in strict compliance with Charles River Laboratories Institutional Animal Care and Use Committee (IACUC) criteria, United States legal regulations on animal welfare, and accepted animal welfare standards.

Experimental design. Following randomization into study groups, animals (5/sex/cohort) received SC doses of RG7652 every other week for 12 weeks (seven total doses) at 0 or 15 mg/kg/week and daily oral gavage doses of atorvastatin at 0, 3, or 20 mg/kg/dose. Blood samples were collected and analyzed for routine hematology, clinical chemistry (including cholesterol and triglycerides) and coagulation (plasma) parameters. Animals were fasted for at least 8 h for clinical chemistry and lipid panel (total cholesterol, HDL- and LDL-cholesterol, and triglycerides) studies. At study termination, all animals were subjected to a complete necropsy evaluation. Weights were recorded for a comprehensive panel of organs, and representative samples of tissues were collected and preserved in 10% buffered formalin. As immunotoxicology was the focus of this study, histopathological evaluation was limited to biopsy of DTH reaction sites, gut-associated lymphoid tissue (GALT), intestine, liver, gallbladder, and axillary as well as mesenteric lymph nodes.

Pharmacokinetics. Serum RG7652 concentrations were measured by a validated enzyme-linked immunosorbent assay (ELISA), using recombinant human PCSK9 for capture and horseradish peroxidase (HRP) conjugated antihuman IgG antibodies for detection. The minimum quantifiable concentration of the assay was 150 ng/ml. Toxicokinetic parameters were estimated using WinNonlin pharmacokinetic software (Pharsight Corp., Mountain View, CA). A noncompartmental approach consistent with the SC route of administration was used for the estimation of maximum serum concentration (Cmax) and area under the concentration versus time curve (AUC) parameters. Anti-RG7652 antibodies were measured by a validated bridging ELISA, using biotin and digoxin labeled RG7652 for capture and HRP conjugated mouse antidigoxin.
antibody for detection. Plasma atorvastatin concentrations were quantified by using high pressure liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) and the results were reported as mean with standard deviation of the mean (Covance Laboratory, Inc., Madison, WI). The lower limit of quantification for atorvastatin in plasma was 0.15 μg/ml.

**Immunological endpoints.** TDAR were monitored following intramuscular (IM) immunization with KLH (Pierce Biotechnology, Rockford, IL) on days 44 and 65 of the study. KLH powder was reconstituted with sterile water for injection at a concentration of 10 mg/ml and injected IM at a dose of 10 mg/animal. Subsequently, serum samples were analyzed by ELISA for anti-KLH IgM and IgG antibodies.

To evaluate DTH reactions, animals were immunized with (1–5) × 10^5 cfu/animal *Mycobacterium bovis* (BCG (Schering-Plough) in a 1:1 emulsion with Incomplete Freund’s Adjuvant (IFA; Pierce Biotechnology, Rockford, IL) prior to initiation of dosing (weeks −5 and −3) and subsequently immunologically challenged with intradermally administered tuberculin (Pierce Biotechnology) or phosphate buffered saline (PBS) on day 83 of the study. Twenty four and 48 h postinjection (days 84 and 85), the dermal sites of administration were scored for degree of induration, erythema, and eschar formation at injection sites. Skin biopsies of the injection sites were collected from all animals and one half of each biopsy sample was collected in Optimal Cutting Temperature (OCT) media (Sakura Finetek, Torrance, CA) and frozen in a dry ice/butane bath for immunohistochemical staining for CD4, CD8, CD31, and CD68. The other half was formalin fixed, embedded in paraffin, and immunohistochemically stained for CD4 and CD31.

NK cell activity in blood was quantified by a calcein-release NK cell cytolytic activity assay using cynomolgus monkey peripheral blood mononuclear cells (PBMCs) as effector cells. NK cell activity was evaluated as the percentage lysis of target cells (K-562) at three effector:target ratios of 100:1, 50:1, and 25:1.

Blood samples (~1 ml) were periodically collected in tubes containing the anticoagulant sodium heparin for peripheral blood immunophenotyping by flow cytometry for monocytes (CD45+/CD14+), B lymphocytes (CD45+/CD20+), T lymphocytes (CD45+/CD3+), T-helper (CD45+/CD3+/CD4+), T-cytotoxic (CD45+/CD3+/CD8+), and NK cells (CD45+/CD3+/CD16+). BD TruCount tubes (BD Biosciences, San Jose, CA) were used in combination with CD45 labeling for real time quantification of absolute counts. Samples were analyzed with a FACSCanto II Flow Cytometer (BD Biosciences) and DIVA 6.0 software.

**RESULTS**

**General Safety**

Administration of RG7652 was well-tolerated following 12 weekly doses of 15 mg/kg RG7652 either alone or in combination with atorvastatin (3 and 20 mg/kg) administered daily by oral gavage. No adverse clinical signs or changes in body weight, food consumption, physical examinations, hematology, coagulation parameters, or urinalyses were attributed to administration of RG7652, atorvastatin or their combination. Similarly, with the exception of lipid parameters discussed below there were no clinical chemistry changes that could be attributed to either drug. Clinical signs prior to BCG immunization (live attenuated mycobacteria) were infrequently observed, but occurred in all dose groups. The BCG injection sites were on the lower region of the back and variably included observations of erythema, swelling, and less frequently abscessation with a supplicative discharge. Other clinical signs related to the procedure specific to our study included erythema, swelling and/or eschar at the abdominal tuberculin intradermal injection sites prior to initiation of dosing (DTH assessments) on week 12 or incision sites associated with the skin biopsies collected during week 12. Animals dosed with 20 mg/kg atorvastatin (3/10 animals dosed with atorvastatin alone and 3/10 animals dosed with atorvastatin combined with 15 mg/kg RG7652) had minimal to mild focal hemorrhages within the mucosa of the colon and this finding may have been related to the administration of atorvastatin; however, similar observations were noted occasionally as a background finding in cynomolgus monkeys used in our study.

**Cholesterol and Triglycerides**

In general, the impact of RG7652 and/or atorvastatin on serum cholesterol and triglycerides was similar between males and females (Fig. 1). Mean serum LDL-c concentrations were decreased in all males and females administered RG7652 (statistically significant in males and females at most time points) between days 8 and 86. For groups receiving 15 mg/kg RG7652, mean LDL-c concentrations on day 86 were 15 and 22 mg/dl (males and females, respectively), which was significantly lower than in control groups (48 and 69 mg/dl, respectively). The effect on LDL-c concentration was dose-dependent in the RG7652/atorvastatin groups with decreases to 11 and 18 mg/dl (males and females, respectively) at 15/3 (RG7652/atorvastatin) mg/kg. For 15/20 mg/kg RG7652/atorvastatin LDL-c decreased to 5 and 7 mg/dl (males and females, respectively). There was a decreasing trend in mean HDL-c concentration in animals administered 20 mg/kg atorvastatin or 15/20 mg/kg RG7652/atorvastatin (statistically significant in males on day 57 for atorvastatin-only group). The magnitude of decrease in these groups was similar and varied from 0.68x- to 0.76x-control in males and from 0.55x- to 0.76x-control in females between days 8 and 86. No effect on HDL-c concentration was observed at 15 mg/kg RG7652 in males or females without atorvastatin or with 3 mg/kg atorvastatin. A consistent decrease in mean triglyceride concentration in both sexes was associated with the combined administration of 15/20 mg/kg RG7652/atorvastatin. Mean triglyceride concentration at this dose combination was decreased to 0.51x- and 0.31x-
FIG. 1. Serum cholesterol (LDL, HDL, and total) and triglycerides. Serum LDL-c was significantly reduced in males (A) and females (B) following administration of RG7652 either alone or in combination with atorvastatin. Differences were statistically significant for nearly all time points ($p < 0.05$). There was a decreasing trend in mean HDL-c concentration (males and females shown in (C) and (D), respectively) in animals administered 20 mg/kg atorvastatin or 15/20 mg/kg RG7652/atorvastatin (statistically significant in males on day 57 for atorvastatin-only group, $p < 0.05$). Effects on total cholesterol are shown in (E) and (F) and effects on triglycerides are shown in (G) and (H) for males and females, respectively. LDL-c: low density lipoprotein-cholesterol; HDL-c: high density lipoprotein-cholesterol.

Pharmacokinetics

Summary (mean ± standard deviation) of $C_{\text{max}}$ and AUC for RG7652 in male and female monkeys receiving SC administration of RG7652 are presented in Table 1. Mean serum drug $C_{\text{max}}$ values were similar for cohorts receiving RG7652 alone and in combination with low dose atorvastatin (260 μg/ml vs. 255 μg/ml, respectively). However, relatively lower mean drug concentrations were observed when RG7652 was administered concomitantly with the high dose atorvastatin (20 mg/kg/day, mean $C_{\text{max}} = 194$ μg/ml). Similarly, exposures AUC (0–14), AUC (0–85), and AUC (0–$t$) were similar for cohorts receiving RG7652 alone and in combination with the low dose atorvastatin but lower when in combination with high dose atorvastatin. Males consistently had slightly higher exposure than the females for all dose groups. Following administration of RG7652,
antitherapeutic antibodies (ATA) were observed in 2 of 10 animals receiving RG7652 alone and in 1 of 10 animals receiving both RG7652 and low dose atorvastatin. All ATA-positive animals were female and there was no apparent reduction in overall RG7652 exposure for the three ATA-positive females. Plasma concentrations of atorvastatin were measured at 2 h postgavage on days 1 and 85 to confirm exposure (Table 1). Mean plasma concentrations of atorvastatin were measured at 2 h postgavage for the three ATA-positive females. Plasma males were female and there was no apparent reduction in overall both RG7652 and low dose atorvastatin. All ATA-positive ani-

**Immunological Evaluations**

**IgM and IgG responses to KLH.** There were no changes in humoral responses as measured by primary and secondary anti-KLH IgM or IgG center point titer (CPT) values, considered to be due to administration of RG7652, atorvastatin or their combination. IgG and IgM responses specific to KLH vaccinations are shown in Figure 2. All animals produced an anti-KLH IgM response after the primary immunization as measured on days 5 through 21 postimmunization.

The secondary immunization on day 65 developed KLH-specific IgM responses similar to those after primary immunization. When the anti-KLH IgM CPT values for the dosed groups were compared with the control group values, statistically significant differences from control values were observed at several time points (day 72 for RG7652 plus low dose atorvastatin and days 61, 70, and 72 for RG7652 plus high dose atorvastatin), however, these increases were not considered to be indicative of an anti-KLH IgM response mediated by RG7652/atorvastatin.

All animals produced an anti-KLH IgG response after the primary immunization as measured on days 5 through 21 postimmunization. Detectable levels of anti-KLH IgG antibodies were observed in all animals by day 51 (7 days postprimary immunization) with mean values increasing further by ~2.5–4-fold by day 54 (10 days postprimary immunization) and an additional 1.2–1.9-fold by day 58 (14 days postprimary immunization). A memory response was observed in all animals as evidenced by ~1.9–4.2-fold increases in mean anti-KLH IgG from days 65 to 72. Comparison of the data obtained from dosed groups to that of the control group showed that there were no statistically significant differences at any time point for the anti-KLH IgG CPT values. When the data was compared with anti-KLH IgM and IgG CPT values for the reference ranges generated from the testing facility, there were no deviations that could be attributed to either RG7652 or atorvastatin.

**NK cell activity.** NK cell activity, as measured by a calcein-release NK cell cytolytic assay, is shown in Table 2. Effector-to-target cell ratios of 100:1, 50:1, and 25:1 were tested for all animals. Administration of RG7652, atorvastatin, or their combination over this range, was not associated with any changes in the NK cell cytolytic activity. Any increases or decreases in NK cell cytolytic activity compared with the predose ranges were con-

**Peripheral blood immunophenotyping.** Administration of RG7652, either alone or in combination with atorvastatin was not associated with changes in the lymphocyte subset and monocyte populations evaluated via flow cytometry (Table 3). Any increases or decreases in lymphocyte subset and monocyte populations compared with the predose average as well as individual predose and control group range were considered to be within the limits of normal variation. There were no statistically significant differences at any time point when the data were expressed either as absolute counts or as percentage of cells gated when compared with the control cohort.

**DTH assessments.** Skin biopsy samples of the tuberculin/PBS injection sites were scored for degree of induration, erythema, and eschar formation and severity scores are summarized in Figure 3. Skin biopsies varied in size, and amount of subcutis present in sections may have influenced severity scores as significant inflammation was graded in both the dermis and subcutis of sections containing both elements. Some sections had increased inflammation in the subcutis compared with the dermis, whereas other sections had no subcutis present in the biopsy specimen. Overall, a similar magnitude of response was observed across dose groups including controls. For animals given RG7652, atorvastatin, or their combination, there was an increase in severity scores from the 24-h biopsy collection time point to the 48-h time point, consistent with expected DTH response due to increasing inflammation with time. In contrast, animals administered vehicle control had slightly lower severity scores at 48 h as compared with the 24-h time points. This difference may reflect earlier resolution of inflammation in the control group compared with those dosed with RG7652, atorvastatin or their combination.

Skin biopsy samples of the tuberculin/PBS injection sites were also evaluated for cellular expression of CD4 (T-helper lymphocytes), CD8 (cytotoxic T-lymphocytes), or CD68 (macrophages) by immunohistochemical methods. Challenge sites on the abdomen were designated as site 1 (PBS-1), site 2 (TB-1), and site 3 (TB-2) and these sites were intradermally injected with PBS at site 1 (PBS-1), undiluted tuberculin at site 2 (TB-1), and TB diluted 1:10 with PBS at site 3 (TB-2) prestudy, and on day 83. Skin biopsies varied in size, and amount of subcutis present in sections may have influenced severity grades as significant inflammation was graded in both the dermis and subcutis of sections containing both elements. Some sections had increased inflammation in the subcutis compared with the dermis, whereas other sections had no subcutis present. In general, the PBS-1 for all animals contained very low or no CD4+, CD8+, and CD68+ cells and responses were comparable across dose groups. There was increased CD4+, CD8+, and CD68+
cell content for TB-1 and TB-2 for all animals in all dose groups compared with PBS-1, consistent with a DTH reaction. Animals given RG7652 plus low dose atorvastatin appeared to have lower CD4+ cell content for TB-1 and TB-2 compared with the other groups; there was a trend toward lower CD8+ and CD68+ cell content for TB-1 and TB-2 in animals dosed with 15 mg/kg RG7652 combined with either 3 or 20 mg/kg atorvastatin when compared with controls or animals dosed with RG7652 or atorvastatin alone. Although lower compared with other dose groups, the severity of DTH responses in groups dosed with RG7652 combined with either 3 or 20 mg/kg atorvastatin were considered to be within normal variability, and immunologically insignificant.
TABLE 2

NK Cell Activity (% Lysis, Mean ± SD)

<table>
<thead>
<tr>
<th>Effector:target ratio</th>
<th>Sex</th>
<th>Time point</th>
<th>Vehicle control</th>
<th>RG7652 15 mg/kg/week</th>
<th>Atorvastatin 20 mg/kg/day</th>
<th>RG7652 + atorvastatin 3 mg/kg/day</th>
<th>RG7652 + atorvastatin 20 mg/kg/day</th>
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<tbody>
<tr>
<td>100:1</td>
<td>Females</td>
<td>Predose</td>
<td>56.1 ± 23.4</td>
<td>67.4 ± 10.5</td>
<td>59.7 ± 17.0</td>
<td>51.7 ± 18.2</td>
<td>58.0 ± 14.6</td>
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<tr>
<td></td>
<td>50:1</td>
<td></td>
<td>51.2 ± 23.3</td>
<td>60.8 ± 10.1</td>
<td>58.0 ± 16.7</td>
<td>50.6 ± 20.4</td>
<td>62.1 ± 12.6</td>
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<td></td>
<td>25:1</td>
<td></td>
<td>50.6 ± 24.0</td>
<td>60.7 ± 10.8</td>
<td>56.9 ± 18.5</td>
<td>47.3 ± 19.6</td>
<td>62.5 ± 11.0</td>
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<tr>
<td>100:1</td>
<td>Females</td>
<td>Day 83</td>
<td>41.3 ± 19.0</td>
<td>50.2 ± 3.33</td>
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<td>32.6 ± 9.07</td>
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<td>50:1</td>
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<td>37.1 ± 19.3</td>
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<td>48.9 ± 13.4</td>
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<td>48.4 ± 15.3</td>
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<td>49.1 ± 18.9</td>
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<tr>
<td>100:1</td>
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<td>52.2 ± 21.3</td>
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</table>

Note. Statistical analysis of the data demonstrated that there were no statistically significant differences in NK cell cytolytic activity between the control group and the dosed groups at any time point (Dunnett, p < 0.05). NK cell: natural killer cell; SD: standard deviation.

FIG. 3. DTH responses following BCG immunization and subsequent tuberculin challenge. Mean severity scores (A) at 24 and 48 h posttuberculin or PBS challenge. Severity grades of 0, none; 1, minimal; 2, mild; 3, moderate; and 4, marked were assigned based on cell content. Severity scores were calculated by severity grade \(\times\)% affected blood vessels in the tissue section rounded to the nearest first decimal point. Scoring for CD4 (T-helper lymphocytes), CD8 (cytotoxic T-lymphocytes), and CD68 (macrophages) positive cells are shown in (B), (C), and (D), respectively. Severity grades of 0, none; 1, minimal; 2, mild; 3, moderate; and 4, marked were assigned based on cell content for each marker. DTH: delayed type hypersensitivity; PBS: phosphate buffered saline.
Subcutaneous administration of RG7652 to cynomolagus monkeys for 12 weeks significantly reduced circulating LDL-c with minimal impact on HDL-c, similar to the response observed in humans (Tingley et al., 2013). These findings are also consistent with other studies in monkeys evaluating therapeutic agents designed to block PCSK9 binding to LDL-r (Chan et al., 2009; Liang et al., 2012; Ni et al., 2010). In contrast, administration of atorvastatin as a single agent at 20 mg/kg/day significantly lowered HDL-c while not lowering LDL-c serum levels relative to control cohorts, in cynomolagus monkeys at pharmacologically relevant serum concentrations. Previous studies with statins in primate models have reported mixed results in terms of statin-induced reductions in LDL-c. Daily administration of a low-dose (10 mg/animal) of rosvastatin for 6 weeks, and a subsequent daily administration of high-dose (20 mg/kg) for 2 weeks did not significantly impact LDL-c or total cholesterol in hypercholesterolemic monkeys, whereas daily administration of 50 mg/kg simvastatin significantly reduced both LDL-c and HDL-c (Liang et al., 2012). In many species, the insensitivity to statin-induced effects on LDL-c may be partly due to a compensatory increase in PCSK9 expression following statin therapy, which has been hypothesized to limit statin ef-
fectiveness in some patients (Dong et al., 2010; Dubuc et al., 2004; Mayne et al., 2008). In support of this theory, we observed a significant increase in serum PCSK9 following administration of 20 mg/kg atorvastatin (data not shown), and 20 mg/kg atorvastatin in combination with RG7652 reduced LDL-c significantly lower than RG7652 alone. Therefore, both agents when combined lower LDL-c better than either agent alone. The atorvastatin-induced increase in PCSK9 expression may also account for the lower overall exposures to RG7652 due to increased target mediated clearance in the two cohorts receiving a combination of RG7652 and atorvastatin, relative to RG7652 alone. Administration of 20 mg/kg/d atorvastatin significantly reduced HDL-c, consistent with other studies with statins in monkeys (Liang et al., 2012) but there was no evidence that this effect was greater due to coadministration of RG7652.

Under the conditions of this study, administration of 15 mg/kg/week RG7652 reduced serum LDL-c to mean levels consistently <20 mg/ml and concurrent administration of 20 mg/kg/day atorvastatin reduced LDL-c to <10 mg/ml, the latter representing a >80% reduction from baseline. Unlike this dramatic reduction in LDL-c, there was no evidence of an impact of RG7652/atorvastatin on immune function as measured by T-cell dependent antibody responses, NK cell activity, immunophenotype, or DTH. Following both primary and booster vaccinations with KLH, robust IgM and IgG responses were evident in all animals and there were no statistically significant differences between cohorts that could be considered to be RG7652- and/or atorvastatin-related. Animals administered 15 mg/kg RG7652 combined with either 3 or 20 mg/kg atorvastatin had subjectively lower DTH scores but these responses were considered within normal variability and the difference was considered immunologically insignificant.

Cellular plasma membranes contain cholesterol-rich subdomains referred to as lipid rafts, which have been shown to play an important role in cellular signaling (Simons and Toomre, 2000) and alterations in cholesterol content of these lipid structures have been associated with altered signal transduction in T lymphocytes and other immune cells (Kabouridis et al., 2000; Surls et al., 2012). Direct disruption of cholesterol architecture in lipid rafts by lowering serum cholesterol levels, either by diet or pharmacologic intervention, has been proposed as one mechanism by which reductions in serum lipid may modulate immune cell function (Chyu et al., 2014). However, under the conditions of our study, marked reductions in serum LDL-c did not translate into meaningful differences in immune function as measured by TDAR, DTH, NK cell function, and peripheral blood immunophenotyping. Although the authors did not directly evaluate changes in lymphocyte membrane cholesterol content, the marked reductions in LDL-c achieved in this study did not result in substantive changes in immune function. This supports a conclusion that lipid raft architecture was not disrupted to the degree necessary for alterations of the immune parameters evaluated in our study and we further hypothesize that other sources of cholesterol (e.g., cellular synthesis, circulating lipoproteins other than LDL-c) may contribute to maintenance of lipid raft integrity. In response to a decline in intracellular sterol levels, there are cholesterol sensors (e.g., sterol regulatory element-binding proteins, liver X receptors) that can regulate either cellular cholesterol production or enhanced LDL-c uptake of endogenous LDL even when circulating LDL-c levels are low (Horton et al., 2003; Zhao and Dahlman-Wright, 2010).

The well-documented effects of statins on immune function do not appear to translate into clinically significant detriments in immune responses to pathogens or vaccines as evidenced by their collective safety profiles (Armitage, 2007; Law and Rudnicka, 2006; Mills et al., 2011; Naci et al., 2013). Statins have been reported to have some suppressive effects on DTH responses, e.g., lovastatin has been shown to decrease Candida albicans-induced inflammatory responses in mice (Mira et al., 2008). Studies characterizing the effects of statins on NK cell activity have shown mixed results. Daily administration of simvastatin in healthy volunteers was shown to reduce NK cell activity by 30% (Hillyard et al., 2004). Lipophilic statins (simvastatin and fluvastatin) but not the more hydrophilic pravastatin have been shown to suppress NK cell cytotoxicity in vitro and this effect was shown to be reversed by adding substrates of isoprenylation but not by the addition of cholesterol (Raemers et al., 2009; Tanaka et al., 2007). However, pravastatin was reported to decrease NK cell activity significantly in patients whereas lovastatin, a highly lipophilic statin, did not (Kobashigawa et al., 1995; Muldoon et al., 1997). In addition to evidence that atorvastatin and other statins can down regulate major histocompatibility (MHC) class II expression on antigen presenting cells, atorvastatin has been shown to enhance T-dependent antibody responses to tetanus toxoid booster vaccination in healthy volunteers (Lee et al., 2006), and simvastatin has been shown to increase the rate of seropositivity following Aβ-immunization in an Alzheimer mouse model (Kou et al., 2010, 2012). In the present study, 20 mg/kg/day atorvastatin administration to cynomolgus monkeys did not significantly reduce NK cell activity, T-cell dependent antibody responses, DTH responses or impact any other evaluated immunological endpoints. This minimal impact on immunomodulatory function is not entirely unexpected in light of the safety record compiled for statins over the last several decades, clinical functional data reported from clinical trials, and preclinical and clinical experimental data on responses to antigen challenges.

In conclusion, the effects of RG7652 in cynomolgus monkeys were limited to the expected and desirable pharmacologic activity of reducing circulating LDL-c. No clinically relevant effects on immune function parameters were identified following administration of RG7652, either alone or in combination with atorvastatin, at exposures consistent with maximal pharmacologic effect.
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