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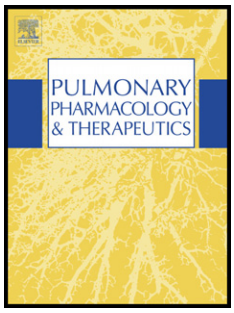

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TLR agonist mediated suppression of allergic responses is associated with increased innate inflammation in the airways

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ABSTRACT

Toll-like receptor (TLR) mediated signalling induces pro-inflammatory responses and can both suppress and exacerbate allergic responses in the airways. The aim of our study was to directly compare the efficacy of different TLR agonists in inhibiting or exacerbating the development of Th2-mediated responses in the airways and investigate if the suppressive effects were associated with increased pro-inflammatory responses. Mice were immunized on day 0, 14 and 21 by intraperitoneal injection of ovalbumin/alum and exposed to ovalbumin aerosol on day 26 and 27. TLR2-, TLR3-, TLR4-, TLR7- and TLR9 agonists (0.001, 0.01, 0.1, or 1mg/kg) were administered intratracheally 1h before each allergen exposure. Both the TLR7- and TLR9 agonists dose dependently reduced airway eosinophilia, while the TLR3 agonist only reduced airway eosinophilia at a dose of 1.0 mg/kg. The TLR2- and TLR4 agonists potentiated eosinophilia. All TLR agonists enhanced neutrophil numbers at doses as low as 0.01 mg/kg, in particular TLR2- and TLR4 agonists. TLR7- and TLR9 agonists also significantly reduced IL-4 and IL-5 levels and all TLR agonists, with the exception of TLR7, enhanced the amount IL-1ß, IL-6, and TNF-α detected in the whole lung lavage. Only application of TLR9 agonist induced detectable levels of IL-10 in the lung. Suppressive effects of the TLR agonists were not dependent upon IFN-γ and IL-10 or associated with increased numbers of $Foxp3^{+}CD4^{+}$ Tr cells in the lavage fluid. Airway resistance was reduced significantly only when TLR7 agonist was administered. When applied therapeutically 2 days after allergen exposure, all TLR agonists, except TLR2, similarly reduced airway eosinophilia and IL-4 levels. Taken together our results show that TLR7 agonists had the strongest anti-asthmatic effects with the lowest pro-inflammatory potential, suggesting that activating TLR7 may have the greatest potential to treat allergic disorders in humans.

Keywords: mouse; asthma; allergy; eosinophils; neutrophils; TLR-agonists; innate-inflammation

1. Introduction

Allergic asthma is characterized by the generation of allergen-specific $CD4^+$ T-helper type 2 (Th2) cells secreting IL-4, IL-5, IL-9, and IL-13 leading to chronic airway inflammation, goblet cell metaplasia, airway hyper-reactivity (AHR) and, in its severest form, tissue remodeling. Currently it is not entirely clear why one person develops asthma and another does not although both are exposed to allergens to the same degree. Genetic and environmental factors clearly have an impact [1,2]. In particular a lack of infections or the lack of exposure to certain commensals during early childhood have been hypothesized to be one, if not the major reason for the steady increase in asthma prevalence observed in both developed and recently developing countries [3-5].

Epidemiological findings and animal experiments support this view, and several studies have tried to identify factors which mediate the observed anti- or pro-asthmatic effects. The most prominent factors identified are Toll-like receptors (TLR). Currently 10 TLRs have been described in humans and 13 in other mammals. They belong to the family of pathogen recognition receptors (PRRs) and are expressed on a variety of cells. They bind to specific molecules (e.g., DNA, RNA, lipopolysaccharides (LPS), lipoteichonic acids (LTA), and others) produced by the pathogen and by commensals. TLR1, TLR2, TLR4, TLR5, TLR6, TLR10 and TLR11 are expressed on the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 are expressed within the cells [6].

Animal studies have shown that the application of certain TLR-agonists can suppress the development of asthma and allergic responses [7-25]. The most widely studied TLR agonists are agonists of TLR2, TLR3, TLR4, TLR7, and TLR9. However, there are also

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studies showing that TLR agonists, in particular endotoxins, promote the development of asthma and that this effect is dependent on the dose administered [26-31].

The aim of our current study was to directly compare the effect of different TLR agonists (TLR2: lipoteichonic acid; TLR3: Poly(I:C); TLR4: lipopolysaccharide; TLR7: resiquimod; TLR9: CpG-oligodeoxynucleotides) on the development of asthmatic responses in murine models of asthma. The most important question we wanted to answer was, which TLR-agonist is most efficacious in reducing allergic responses at a dose where no or very little innate inflammation is induced. We found that the different TLR agonists tested have different suppressive effects on the development of Th2-responses and different proinflammatory properties in the airways of mice and these depend on the dose and in which setting they were used. Reduction of Th2 responses, with the exception of using a TLR7 agonist, was always associated with increased proinflammation.

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2. Materials and methods

2.1 TLR agonists

For activation of murine TLR2, TLR3, TLR4, TLR7 and TLR9 the respective agonists were used; lipoteichonic acid from *Staphylococcus aureus*; LTA-SA, synthetic analog of double stranded RNA; Poly(I:C), lipopolysaccharide from *E.coli K12*; LPS-EK, small synthetic antiviral imidazoquinoline compound; R-848 and synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides; ODN1826. All TLR-agonist were purchased from InvivoGen, San Diego, USA.

2.2 Mice

Female C57Bl/6, IL10-/- deficient (C57Bl/6 background), and BALB/c mice were purchased from Charles River (Sulzfeld, Germany). At the onset of the experiments, animals were between 8 and 12 weeks of age and were kept in a pathogen-free animal facility. The mice had free access to food and water. Care and use of experimental animals conformed to the 'Guide for the Care and Use of Laboratory animals' published by the National Institute of Health (NIH publication 85–23, revised 1985), and the study was approved by the local governing authority.

2.3 Treatment protocols

C57Bl/6, IL10-/- deficient (C57Bl/6 background) and BALB/c mice were sensitized intraperitoneal (i.p.) with a mixture of 20 µg ovalbumin (Serva, Heidelberg, Germany) solved in 100 µl phosphate buffered saline (PBS, Biowhittaker, Lonza, Verviers, Belgium) and adsorbed to 100 µl $Al(OH)_3$ (Alum, Pierce, Rockford, USA). The total volume of 200 µl was administered to animals on day 1, 14 and 21. Mice were exposed to 1% ovalbumin or PBS aerosol on two consecutive days (day 26 and day 27) for 20 min. In the protective models

0.001, 0.01, 0.1, and 1.0 mg/kg of TLR2-, TLR3-, TLR4-, TLR7- or TLR9 agonists (InvivoGen, San Diego, USA) per mouse was solved in 50 µl PBS and administered intratracheal (i.tr.) 1 h before each ovalbumin exposure (Fig. 1A) or 1 mg/kg 4 days prior to allergen exposure (Fig. 1B). In the therapeutic model 1 mg/kg of respective TLR agonist was administered intratracheal on day 29 followed by an additional ovalbumin exposure on day 33 (Fig. 1C). Mice were sacrificed 24 h after the last ovalbumin exposure. Vehicle controls were sensitized intraperitoneal with PBS and Alum, and were exposed to PBS aerosol. The ovalbumin and vehicle controls all received a sham intratracheal application of 50 µl PBS at the same point of time the other groups received TLR agonists.

2.4 Treatment of Mice with anti–IL-10 receptor and anti-IFNγ neutralizing antibodies

Anti–IL-10 receptor (α–IL-10R) monoclonal Antibodies (mAbs) (1B1.2) and anti-IFNγ (α-IFNγ) mAbs (XMG1) were generously provided by Prof. Dr. Edgar Schmitt (Mainz, Germany). BALB/c mice were treated with anti–IL-10R or anti-IFNγ mAbs (250 µg/mouse intraperitoneal). As controls, mice were treated with 250 µg/mouse of purified rat serum IgG (control IgG) mAbs (Sigma, Steinheim, Germany). All antibodies were administered on day 25, 1 day prior to TLR application and ovalbumin exposure.

2.5 Whole lung lavage

Whole lung lavage was performed at the indicated time-points, 24 h after the last ovalbumin exposure. Lavage buffer consisted of 1 % bovine serum albumin (Sigma-Aldrich, Steinheim, Germany), 0,0012 % 0,5M EDTA (Promega, Madison, USA) and proteinase inhibitor (Complete Protease Inhibitor Cocktail Tablets, Roche Diagnostics GmbH, Mannheim, Germany) in Hank´s salt solution (Biochrom AG, Berlin, Germany) After insertion of a cannula into the trachea lungs were flushed with 0.8 ml of buffer twice.

2.6 Detection of Cytokines by ELISA and multiplex technology

In the protective model (Fig. 1A) amount of cytokines and chemokines in the whole lung lavage fluid were determined using the 96 well plate mouse cytokine/chemokine premixed multiplex lincoplex KIT assay (LINCO, St. Charles, USA) according to the manufactures instructions. In brief, fluorescently labeled microspheres coated with cytokine-specific capture mAbs were incubated overnight at 2-8° C with 25 µl of whole lung lavage. After two washing steps, biotinylated detection mAbs were added and incubated for 60 min at room temperature, followed by 30 min of incubation with streptavidin-phycoerythrin. After three washing steps sheath fluid was added and the plates were analyzed using a Bio-Plex reader (Bio-Rad, München, Germany). Standard curves and concentrations were calculated with BioPlex Manager 3.0 software. In preventive and therapeutic models amounts of IL-4 and IL-5 in the whole lung lavage were measured by standard ELISA, using BD Biosciences mouse IL-4 and BD Biosciences mouse IL-5 ELISA sets (BD Biosciences, San Diego, USA) according to the manufactures instructions for detection.

2.7 Histological analysis

Lungs were prepared 24 h after the last TLR ovalbumin exposure. Lungs were fixed in 4% phosphate-buffered formalin for 24 h and embedded in paraffin wax. Sections (2-3 µm) were cut and stained using standard histological protocols with haematoxylin and eosin (H&E) reagent (Merck, Darmstadt, Germany). The stained sections were visualized by light microscopy.

2.8 Measurement of airway hyperreactivity

For invasive measurements of resistance mice were anesthetized with 1.8 % halothane/40 % oxygen by inhalation after pre-medication with 77 mg/kg propofol given intraperitoneal on day 28, 24±2 h after the final ovalbumin exposure. AHR was then assessed

after challenge with aerosolized methacholine chloride (MCh; Sigma, Deisenhofen, Germany) in intubated, spontaneously breathing mice as described previously [32,33]. Briefly, mice were placed in supine position in a body plethysmography (type 871, HSE-Harvard Apparatus, March-Hugstetten, Germany) and pulmonary resistance (RL) was calculated from the measured signals of transpulmonary pressure and tidal respiratory flow and continuously recorded using commercial software (HEM 3.5, Notocord, Croissy, France). After recording of baseline values, the mice were exposed to aerosolized MCh (from 5 % solution) in dose steps of 0.063, 0.125, 0.25, 0.5, 1, 2 and 4 µg under continuation of lung function registration. Dried aerosols were generated by a computer-controlled, jet-driven aerosol generator system (particle size 2.8 µm MMAD; Bronchy III, Fraunhofer ITEM, licensed by Buxco, Troy, NY, USA). Aerosol concentrations were determined by a gravimetrically calibrated photometer. The total dose inhaled via the orotracheal tube in µg was calculated and controlled by a computerized dose-control system (Fraunhofer ITEM) based on the continuously measured respiratory minute volume and aerosol concentration [34]. From dose-effect plots (delta% RL vs. MCh dose) the effective inhalation dose in µg MCh required to produce a 150% increase in RL was evaluated for each animal (ED150 RL).

2.9 Detection of FoxP3^{+} cells by flow cytometry

Whole lung lavage cells were pooled. ACK Lysis Buffer (Lonza, Basel, Switzerland) was used for red blood cell lysis and cells were washed and further processed in PBS supplemented with 0,5% BSA. The cell surface was stained with PerCP-Cy5.5 conjugated anti-mouse CD4 antibody (BD Biosciences, Heidelberg, Germany). For detection of intracellular FOXP3 antigen, the Mouse/Rat FOXP3 staining set (eBioscience, San Diego, USA) was used according to the manufactures instructions. Fluorescence was analyzed on a LSRII Cytometer (BD Biosciences, Heidelberg, Germany) and quantification was performed

by BD FACS DIVA Software 5.0. Gates were set on lymphocytes based on forward and side scatter properties, quadrants were defined using isotype controls.

2.10 Statistical analysis

Statistical differences between different groups were evaluated by One-way ANOVA or unpaired t-test. One-way analysis of variance together with the Dunett´s post test was used for comparisons of all groups vs. the ovalbumin group. In airway hyperreactivity measurement statistical differences of area under the curve (AUC) between vehicle control group and ovalbumin treated animals were evaluated with unpaired t-test. The results are expressed as mean±standard error of means (SEM). A *P*-value of < 0.05 was considered significant.

3. Results

3.1 Effects of TLR agonists in a preventive setting on the development of allergic inflammation in the lungs

The pro-inflammatory and anti-asthmatic effects of five different TLR agonists were tested in a preventive model for allergic asthma in mice. For this purpose, doses of 0.001, 0.01, 0.1 and 1.0 mg/kg of TLR2-, TLR3-, TLR4-, TLR7- and TLR9 agonists were given intratracheal one hour before each allergen exposure (Fig. 1A). We then analyzed which TLR agonists induced the strongest influx of macrophages and neutrophils and which had the strongest inhibitory effect on the development of airway-eosinophilia. In our model a very strong eosinophilia is induced. However, a weak neutrophilia is also present. How the neutrophilia impacts on lung pathology is not known. Based on publications showing that the presence of neutrophils correlates with impaired lung function, we suggest that the neutrophils

together with the eosinophils may contribute to the increased AHR after MCH challenge in the OVA treated and challenged mice. Figure 2 shows that all agonists, with the exception of TLR7 agonist, dose dependently increased the amounts of total macrophages found in the whole lung lavage. A similar effect was seen on the recruitment of neutrophils into the lung. Interestingly, administration of TLR7 agonist also induced a very weak neutrophilia, at a dose of 0.1mg/kg. Surprisingly, compared to other published reports [12;17;24;25], we found that only TLR3-, TLR7-, and TLR9 agonists suppressed the development of allergen induced airway eosinophilia. In contrast, we detected a significant increase in the recruitment of eosinophils when lower doses of either TLR2- or TLR4 agonists were used. Histological analysis confirmed the overall lower inflammation detected in the TLR3-, TLR7- and TLR9 agonist treated mice in comparison to the ovalbumin controls and TLR2- and TLR4 agonist treated animals which showed the strongest inflammation (Fig. 3).

All tested agonists, again with the exception of TLR7, induced the production of the pro-inflammatory cytokines and chemokines IL-6, IL-1ß, TNF-α and reduced the levels of IL-4 and IL-5 in the whole lung lavage (Fig. 4). Most of the observed effects were dose dependent. Interestingly, we found that TLR9 agonist was the only agonist inducing the production of IL-10. IL-13 was also reduced in the mice treated with agonists TLR9, TLR7, TLR4 and TLR2 and no difference in the amount of IFN-γ was found in any of the ovalbumin treated groups (data not shown). IL-12 and IFN-α and -ß could not be detected in any of the whole lung lavage samples. When the TLR agonists were administered alone and the animals were not treated with ovalbumin enhanced IL-6, IL-1 ß, and TNF-α levels were also detected in the whole lung lavage (data not shown).

We also analyzed which of the TLR agonists had suppressive effects on the development of AHR. Figure 5 shows that only the TLR7 agonist significantly reduced AHR.

Both TLR2- and TLR4 agonists have been shown to be able to reduce airway eosinophilia in mice [17;24;25]. We did not see this effect. However, this may be due to

different protocols used. For this reason we also administered the different TLR agonist intratracheal at the highest dose, once, 4 days before the allergen exposure (Fig. 1B). We found that in this experimental setting all of the TLR agonists inhibited the development of airway eosinophilia. No significant increase in neutrophil numbers was detected and TLR4 agonist was the only TLR agonist that induced a significant increase in macrophage numbers at this time point. The lack of neutrophilia was somewhat surprising, since we observed a strong neutrophilia in the airways, when the TLR agonists were administered directly before the allergen challenge (Figure 2.). The most likely explanation for this finding is that in all cases a neutrophilia is induced, however, at the later time-point no longer detectable. Supporting this view are kinetic studies showing that the maximum neutrophilia in the lung is observed 24h after TLR application and no or few neutrophils are present 5 days after the application (data not shown). TLR2-, TLR3-, and TLR4 agonists also reduced IL-4 and IL-5 levels whereas TLR9 agonists only reduced IL-4 levels in the whole lung lavage (Figure 6B). Interestingly, the TLR-4 and TLR9 agonist treated mice also showed reduced AHR (Fig. 6C).

3.2 Suppressive effects of TLR agonists in a preventive setting were not dependent on IL-10, IFN-γor enhanced numbers of Tr cells in the airways

Previous studies suggest that the suppressive effects of different TLR agonists on the development of allergic responses may be associated with the production of IFN-γ, IL-10 or activation of FoxP3+ Tr cells [6;11;13;14;21;34]. For this reason, we repeated the experiments shown in Figure 1A in IL-10 deficient mice on a C57Bl/6 background using the three agonists, which showed a significant reduction in Th2 responses in the lung. Figure 7A shows that the suppressive effects of the three tested TLR agonists were similar in both the control and IL-10 deficient mice. Furthermore, treating ovalbumin immunised/exposed and TLR agonist treated BALB/c mice with anti-IFN-γ or anti-IL-10 receptor neutralizing

antibodies showed that the suppressive effects appeared not to be dependent upon either IFN-γ or IL-10 (Fig. 7B). We also analyzed if increased numbers of $Foxp3^+$ Tr cells could be found in the whole lung lavage of ovalbumin and TLR agonist treated mice. Figure 8 shows that the suppressive effects of TLR3-, TLR7- and TLR9 agonist were not associated with increased numbers of $FoxP3^+CD4^+$ T cells in the whole lung lavage. Interestingly, both TLR2- and TLR4 agonists treated mice showed increased numbers of Tr cells in the whole lung lavage, albeit without reducing the overall numbers of eosinophils. Surprisingly we found strongly reduced numbers of $FoxP3^+CD4^+$ and $CD4^+$T cells in the whole lung lavage of TLR7 agonist treated mice.

3.3 TLR agonist mediated suppression of allergen-induced eosinophilia in a therapeutic setting

The experiments above clearly show that some but not all of the tested TLR-agonists reduced the development of allergen-induced Th2-responses in the lung when administered before allergen exposure. In this experimental preventive setting no lung inflammation has occurred prior to the treatment with the TLR agonists (data not shown). For this reason we also analyzed the effects of the different TLR agonists in a therapeutic setting where the TLR agonists were administered intratracheal 2 days after the second ovalbumin exposure (day 30) followed by a final allergen-exposure on day 33 (Fig. 1C). For these experiments we used the dose which had the greatest suppressive effect in the protective model. Figure 9A shows that all the TLR agonists with the exception of the TLR2 agonist significantly reduced the development of airway eosinophilia. No increase in neutrophil numbers was observed in any of the treated mice. However, TLR4 agonist treated mice showed a significant increase in total macrophage numbers. All TLR agonists reduced IL-4 levels in the whole lung lavage. IL-5 levels were also significantly reduced (with the exception of the TLR4 agonist treated mice).

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4. Discussion

The aim of this study was to determine the efficacy of different TLR agonists in suppressing the development of Th2-mediated responses in the airways and to investigate if the suppressive effects were associated with increased pro-inflammatory responses. We found that when the different agonists were administered intratracheally 1h before allergen exposure (preventive setting) TLR9-, TLR7-, and TLR3 agonists dose dependently suppressed the development of allergen induced airway eosinophilia, and IL-4 and IL-5 levels detected in the whole lung lavage. These findings are in unison with previously published reports [10;13;14;22]. With the exception of TLR7 agonist all the agonists, dose dependently increased the amounts of total macrophages, neutrophils and pro-inflammatory cytokines found in the whole lung lavage. The stronger the inflammatory response induced by TLR9- and TLR3 agonist, the stronger the suppressive effect on the development of allergen-induced Th2-mediated responses. TLR7 agonist showed only a weak induction of a pro-inflammatory response but was the only agonist found to significantly reduce AHR. In contrast to other published reports [12;17;24;25] we found that application of TLR4- or TLR2 agonist did not suppress the development of allergen induced Th2-responses in the lung or AHR. However, this may be due to different protocols used. Supporting this view was our finding that when we administered the different TLR agonist intratracheal at the highest dose once 4 days before the allergen exposure, all of the TLR agonists inhibited the development of airway eosinophilia. This clearly suggests that the effects of TLR4- and TLR2 agonist depend on the time point of application. Supporting this view was our finding that under these conditions the TLR4- and now also the TLR9 agonists reduced AHR, an effect not seen when they were administered directly before allergen challenge. Surprisingly, the TLR7 agonist which showed a protective effect on AHR given directly before allergen challenge, no longer had an effect

under these conditions. This shows that the protective effects of different TLR agonists on AHR strongly depend on the time they were administered in respect to allergen challenge.

It appears that the kinetics of the induction of protective factors were different between the TLR agonists. We detected increased amounts of FoxP3+ cells in TLR2- and TLR4 agonist treated lungs. Possibly these cells need to be activated longer before allergen challenge to have a suppressive effect on the Th2 responses in the lung. A further surprising result was, that TLR agonist induced eosinophil reduction did not always correlate with a reduction in IL-4 and IL-5 (TLR7- and TLR9 agonist) levels in the lavage fluid. Why the effects are so different between the TLR agonists used, although they signal through very similar signal transduction pathways leading to similar proinflammatory responses [6;23], is not clear. We hypothesize that the outcome in respect to the strength and quality of the innate response and the anti-asthmatic effects strongly depend on the expression pattern of the different TLRs on a particular cell and the amount of this cell type present in the lung.

Which mechanisms were responsible for the TLR9-, TLR3- and TLR7 agonist induced reduction of allergic responses in the lung? Based on previous publications the following mediators may play a role IL-10, IL-12, IFN-α and -ß, or IFN-γ. Experiments performed in IL-10 deficient mice or mice treated with anti-IL-10 receptor or IFN-γ neutralizing antibodies suggest that IL-10 or IFN-γ were not responsible for the observed inhibitory effects. This was somewhat surprising for the TLR9 agonist, since we found a strong induction of IL-10 after the intra tracheal application. It is also possible that TLR9- and TLR3 agonist exert their suppressive effect through both IL-10 and IL-12, a mechanism recently published for these two agonists [14]. Type 1 IFNs are strong inhibitors of allergic responses [6;23;34;35], however, we could not detect IFN-α or -ß in the whole lung lavage suggesting that these may not be responsible for the suppression of the allergic response. We cannot rule out the possibility that they were induced in too small an amount to be detected by ELISA. The

induction of regulatory T cells (Tr) has also been hypothesized in TLR agonist mediated suppression of allergic disorders [23;34;35]. However, in our experiments $CD4^+FoxP3^+$ T cell numbers in the whole lung lavage did not increase in the groups which showed reduced allergic responses. Interestingly, Tr numbers were increased in the TLR4- and TLR2 agonist treated mice, which showed no reduced allergic response when administered directly before the allergen-exposure.

Currently, we cannot answer the question by which mechanism the different TLR agonists mediate their suppressive effects, however TLR7 agonists may have a different mode of action than the TLR9- and TLR3 agonists. We found, that the TLR7 agonist treated mice had a strong reduction in total $CD4^+$ T cell numbers in the whole lung lavage. This suggests that TLR7 agonist treatment may lead to a loss or lack of recruitment of $CD4^+$ cells into the lung, thus explaining the reduced Th2-responses detected in the airways. Supporting this view is the previously published report that the TLR7 agonist R-848, which we also used for our study, leads to a lymphopenia [36]. This would also possibly explain why the weak proinflammation detected in the TLR7-agonist treated mice was associated with suppressed Th2-responses in our model. If this is a general effect of a TLR7 agonist or an off-target effect of R-848 needs to be determined.

When treating patients with atopic asthma, with the exception of seasonal asthma, all patients will have ongoing Th2 type inflammation in the airways [1]. In respect to possible future use in humans, we also analyzed the effects of the different TLR agonists in a therapeutic setting. In this setting the TLR agonists were administered after the allergic inflammation in the lung has already occurred. We found that all TLR agonists with the exception of TLR2 significantly reduced the development of airway eosinophilia and IL-4 levels in the whole lung lavage. No increase in numbers of neutrophils was observed. This

suggests that TLR agonists may also be used to treat patients with established allergic inflammation in the airways.

Previous reports suggest that infections and the application of TLR agonists can also exacerbate allergic responses, in particular TLR4- and TLR2 agonist LPS and LTA-SA [26-29]. We also found some evidence that TLR agonists increased the allergic Th2 response. At low doses both TLR4- and TLR2 agonist significantly increased allergen-induced recruitment of eosinophils into the airways when administered directly into the lung before allergen exposure. This supports the hypothesis, that some TLR agonist can also increase allergic responses, in particular, when low doses are administered. This also needs to be taken into account when patients are to be treated.

In conclusion, we found that the TLR2-, TLR3-, TLR4-, TLR7-, and TLR9 agonists have different suppressive effects on the development of Th2-mediated responses in the airways. These depend on the dose and at which point in time, in respect to allergen exposure, they were given e.g. one day or 4 days before allergen challenge or in a therapeutic setting after allergen challenge. All the tested agonists induced airway neutrophilia and with the exception of TLR7 agonists increased the amounts of pro-inflammatory cytokines in the airways, albeit to different degrees, when directly administered into the lung. This raises the question if any of the tested TLR agonists are safe for human use when repeatedly administered directly into the lung. In particular TLR2- and TLR4 agonists which showed a severe inflammation in the lungs as detected by histology and the presence of large numbers of neutrophils in the lung lavage. However, the TLR7 agonists showed only a weak pro inflammatory potential and a high anti-allergic effect, suggesting that a TLR7 agonist may be used in humans to treat asthma. Nevertheless, it is possible, that small pro-inflammatory responses may be tolerable in patients suffering from allergic asthma, thereby possibly also enabling the use of other TLR agonists. However, there may be special safety issues regarding

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the use of novel therapeutic agents that might cause an increase in neutrophils in a population of patients with already altered airway function.

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6. FIGURE LEGENDS

Fig. 1: Treatment protocols used for TLR agonist administration. (A) Protective model with administration of TLR agonists on day 26 and 27, **(B)** protective administration of TLR agonists on day 22, and **(C)** therapeutic model with administration of TLR agonists on day 30.

Fig. 2: TLR agonists suppress allergen-induced eosinophilia and induce airway neutrophilia. (A) Total number of cells in whole lung lavage was determined 24 h after the last ovalbumin exposure. Data are presented as mean±SEM of 8 mice/group. *p<0.05, **p<0.01, ***p<0.001, in comparison with the ovalbumin group.

Fig. 3: Histological analysis of the lungs from ovalbumin and TLR agonist treated mice. **(A)** Lung tissues were obtained from naïve mice treated with the TLR agonists alone or **(B)** from ovalbumin sensitized and exposed mice treated with different TLR agonists as indicated in Figure 1 (A) (1.0 mg/kg of TLR2-, TLR3-, TLR4-, TLR7- and TLR9 agonist were used). Tissues were stained with haematoxylin and eosin, and examined by light microscopy. Scale bar = 100µm. Shown are representative examples of 8 mice/group.

Fig. 4: TLR agonist treated mice show a reduction of Th2 cytokines and increase in pro-inflammatory cytokines and chemokines in the airways. Mice were sensitized and challenged with ovalbumin as indicated in material and methods (Fig. 1A). TLR agonists in dose of 1.0, 0.1, 0.01 and 0.001 mg/kg were administered on day 26 and 27. The amount of the different mediators in the whole lung lavage were determined 24 h after the last ovalbumin exposure, as described in materials and methods (■ = TLR2 agonist, ■ = TLR3 agonist, ■ = TLR4 agonist, ■ = TLR7 agonist, ■ = TLR9 agonist). Data are presented as mean±SEM of 8 mice/group. *p<0.05, in comparison with the ovalbumin group.

Fig. 5: TLR7 agonist reduces airway resistance after methacholine exposure. For invasive measurement of resistance mice were first exposed to nebulised saline (PBS), followed by increasing doses of methacholine. Columns represent the effective inhalational dose of MCh in µg required to increase pulmonary resistance (RL) by 150% above baseline (ED150 RL). Significant, complete inhibition of airway hyperresponsiveness was observed in mice pre-treated with the TLR7 agonist. Data are presented as mean±SEM with 9-16 mice/group. ***p<0.001, in comparison to the ovalbumin group.

Fig. 6: Pre-treatment with TLR agonists inhibits influx of eosinophils, production of IL-4 and TLR4- and TLR9 agonist pre-treatment reduces airway resistance. BALB/c mice were sensitized with ovalbumin, treated with different TLR agonists and exposed to ovalbumin as indicated in material and methods (Fig. 1B). **(A)** Whole lung lavage was collected 24 h after the last ovalbumin exposure and the number of inflammatory cells and **(B)** levels of IL-4 and IL-5 in the whole lung lavage were determined. **(C)** Invasive measurement of airway resistance. Columns represent the effective inhalational dose of MCh in µg required to increase pulmonary resistance (RL) by 150% above baseline (ED150 RL). Significant inhibition of airway hyperresponsiveness was observed in mice pre-treated with TLR4- and TLR9 agonist. Data are presented as mean±SEM with 6-8 mice/group. *p<0.05, **p<0.01, ***p<0.001 in comparison to the ovalbumin group.

Fig. 7: IL-10 and IFN-γ mediated signaling is not necessary for TLR agonist mediated suppression of airway eosinophilia. **(A)** C57Bl/6NCrl, C57Bl/6NCrl IL10-/-, and **(B)** BALB/c mice were sensitized and exposed to ovalbumin and treated with different TLR agonists as indicated in material and methods (Fig. 1A). **(B)** On day 25 BALB/c mice received intra peritoneal injection of either anti-IL-10R or anti-IFN-γ neutralizing antibodies.

Number of cells in the whole lung lavage was determined 24 h after the last exposure. Data are presented as mean±SEM of 8 mice/group. *p<0.05, **p<0.01, ***p<0.001, in comparison with the ovalbumin group.

Fig. 8: Detection of FOXP3+/CD4+ T-cells in the whole lung lavage from ovalbumin and TLR agonist treated mice. Whole lung lavage cells were stained as described in materials and methods. **(A)** Shown are representative examples of whole lung lavage cells stained for CD4+ surface and FOXP3 intracellular expression. Total numbers of CD4+ and CD4+/FOXP3+ cells in the whole lung lavage (% of ovalbumin control) are shown in **(B)** and **(C)**, respectively. Data are presented as mean±SEM of 4 samples/group (whole lung lavage cells from 4 mice/group were pooled (n=2/group) from 2 separate experiments (total of n=4). *p<0.05, **p<0.01, ***p<0.001, in comparison with the ovalbumin group.

Fig. 9: TLR agonists suppress the development of allergen-induced eosinophilia and IL-4 production in the airways in a therapeutic setting. BALB/c mice were sensitised, exposed to ovalbumin and treated with different TLR agonists as indicated in material and methods (Fig. 1C). **(A)** Whole lung lavage was collected 24 h after the last exposure and the number of inflammatory cells and **(B)** levels of IL-4 and IL-5 in the whole lung lavage were determined. Data are presented as mean±SEM of 8 mice/group. **p<0.01, ***p<0.001, in comparison with the ovalbumin group.

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Figure 1

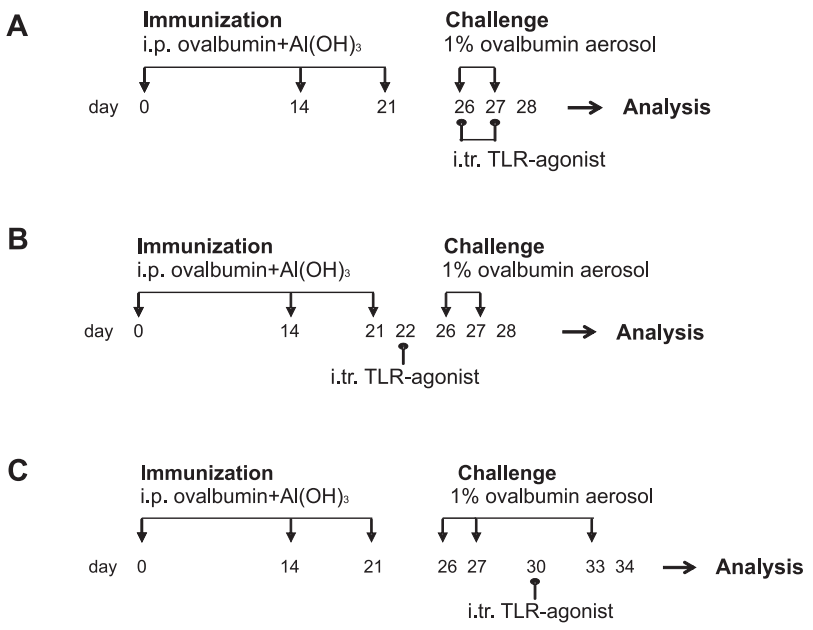

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Figure2

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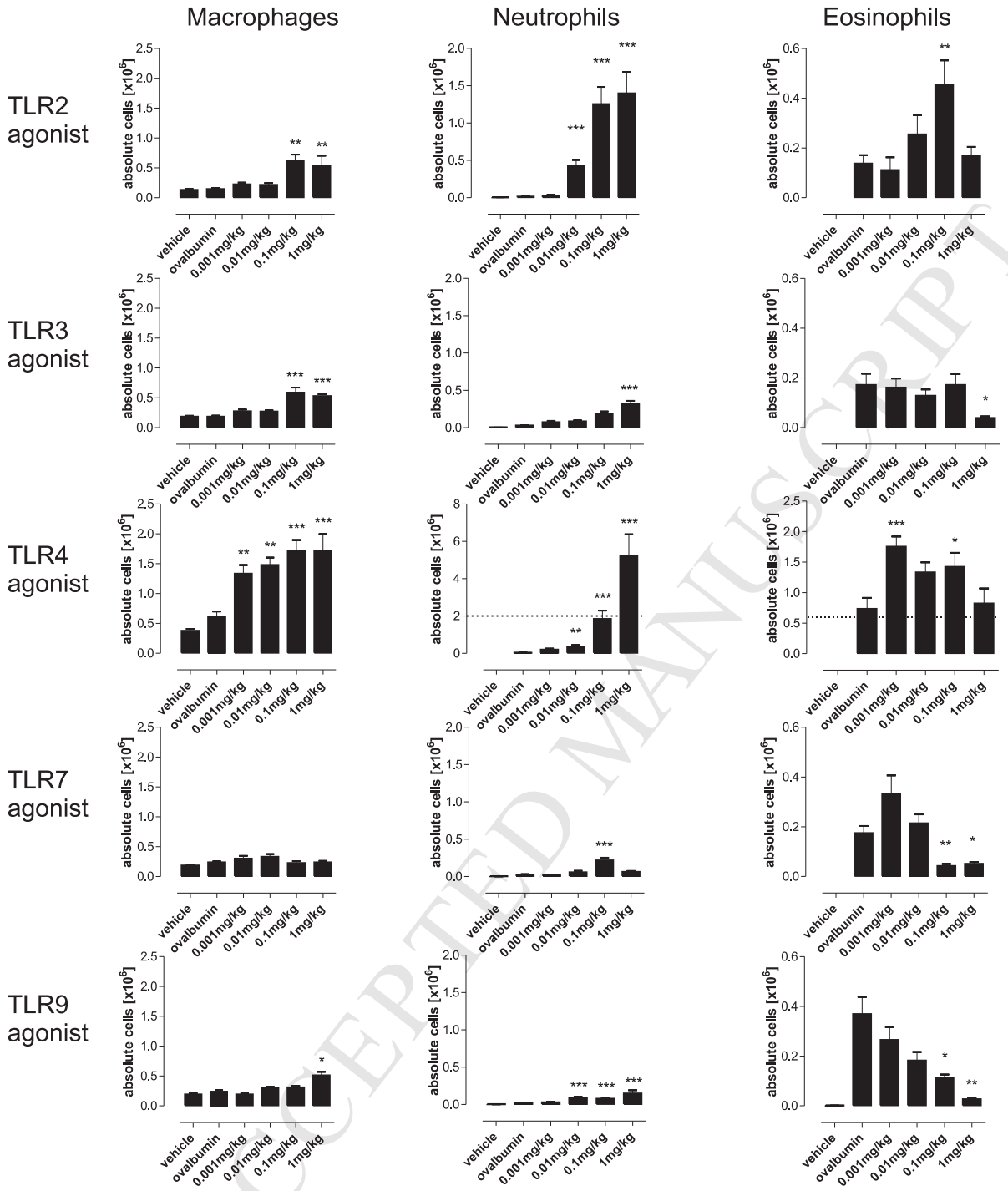
Figure 2



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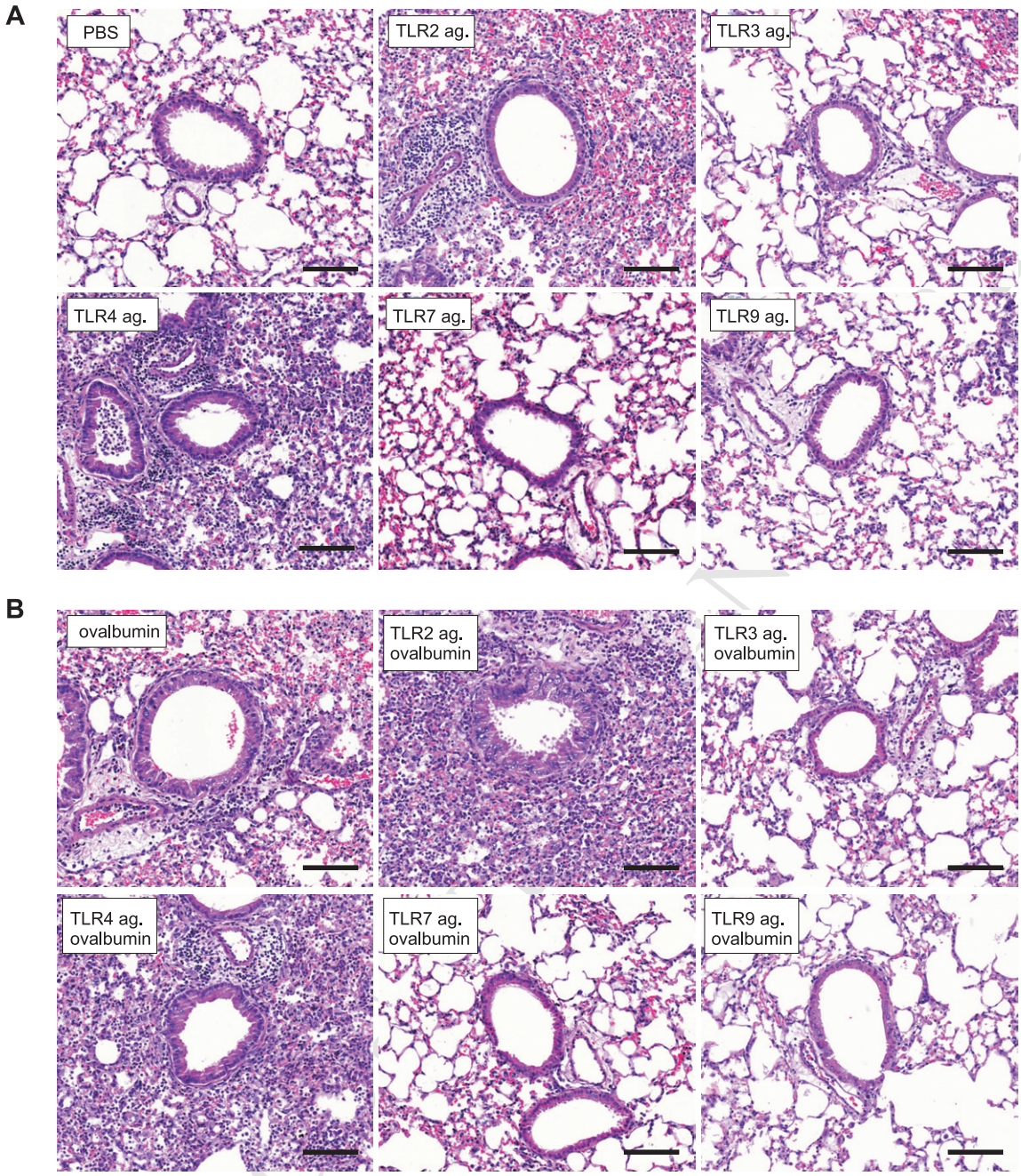

Figure4

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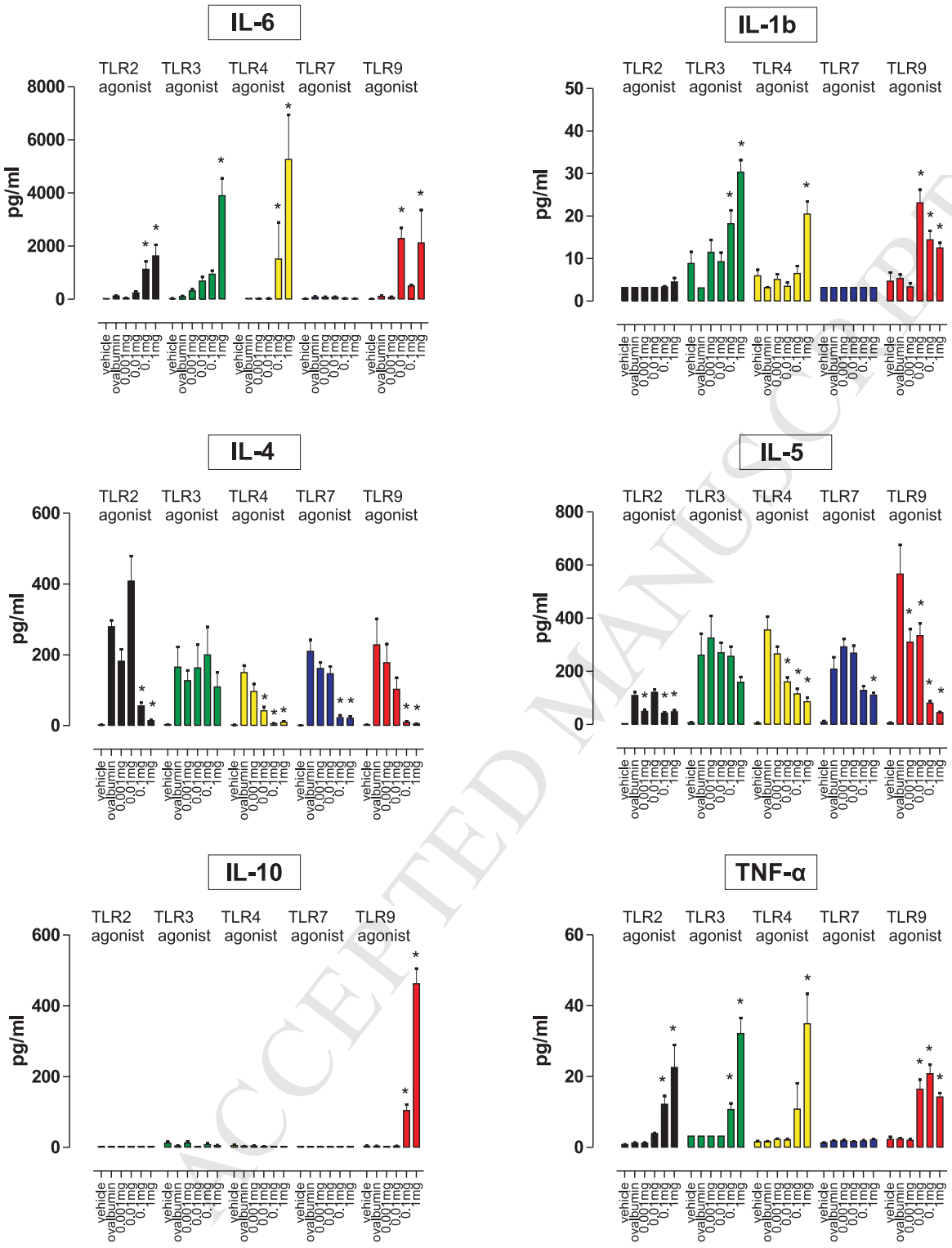
Figure 4



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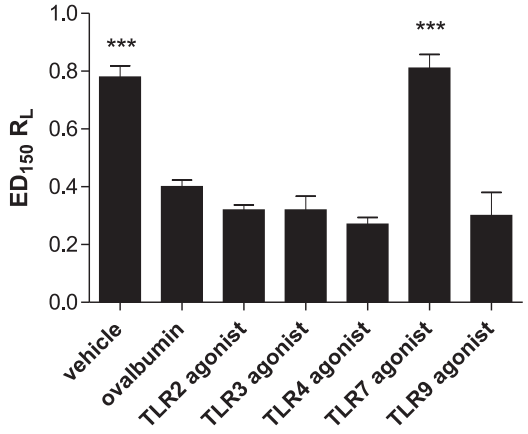

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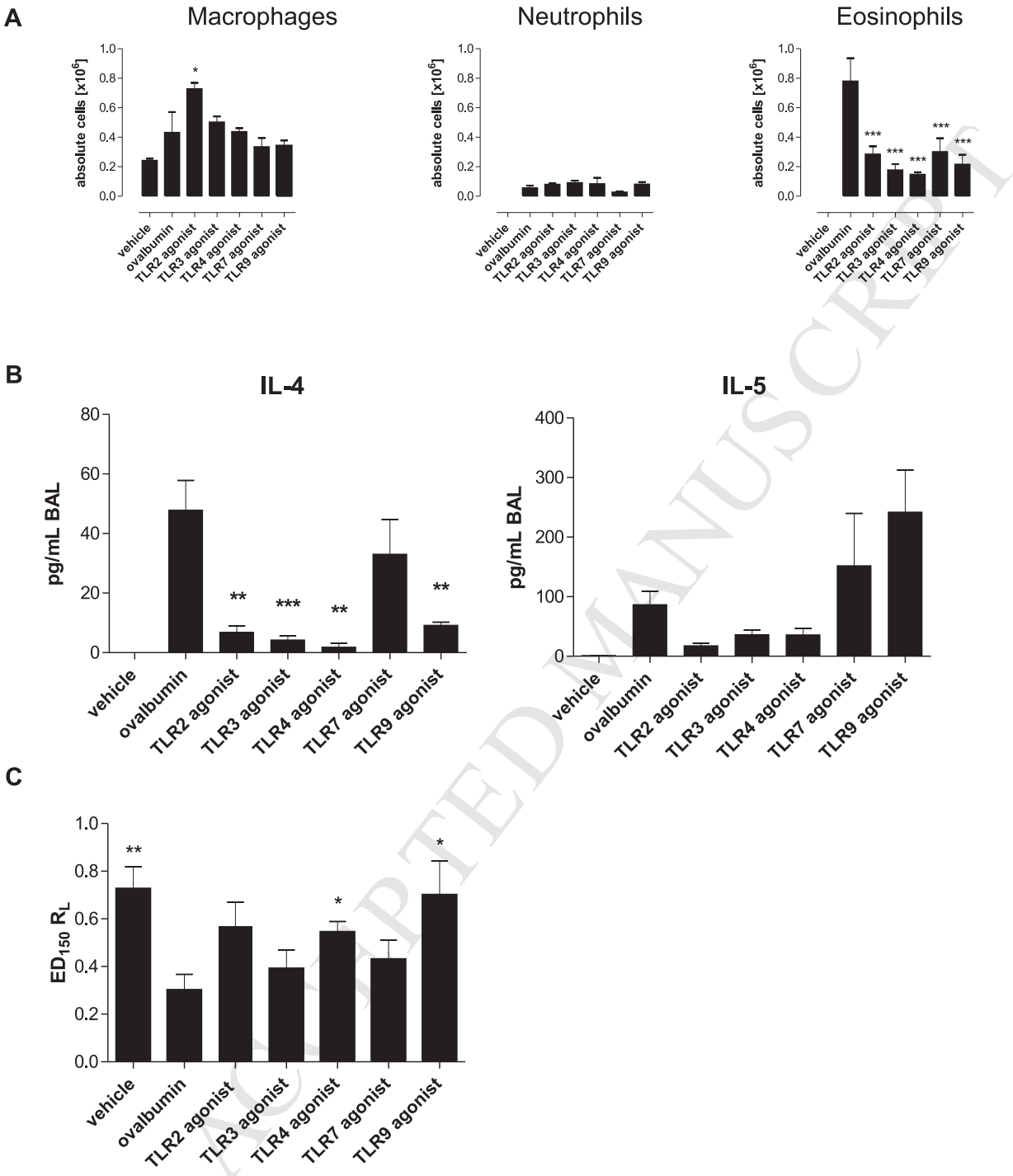

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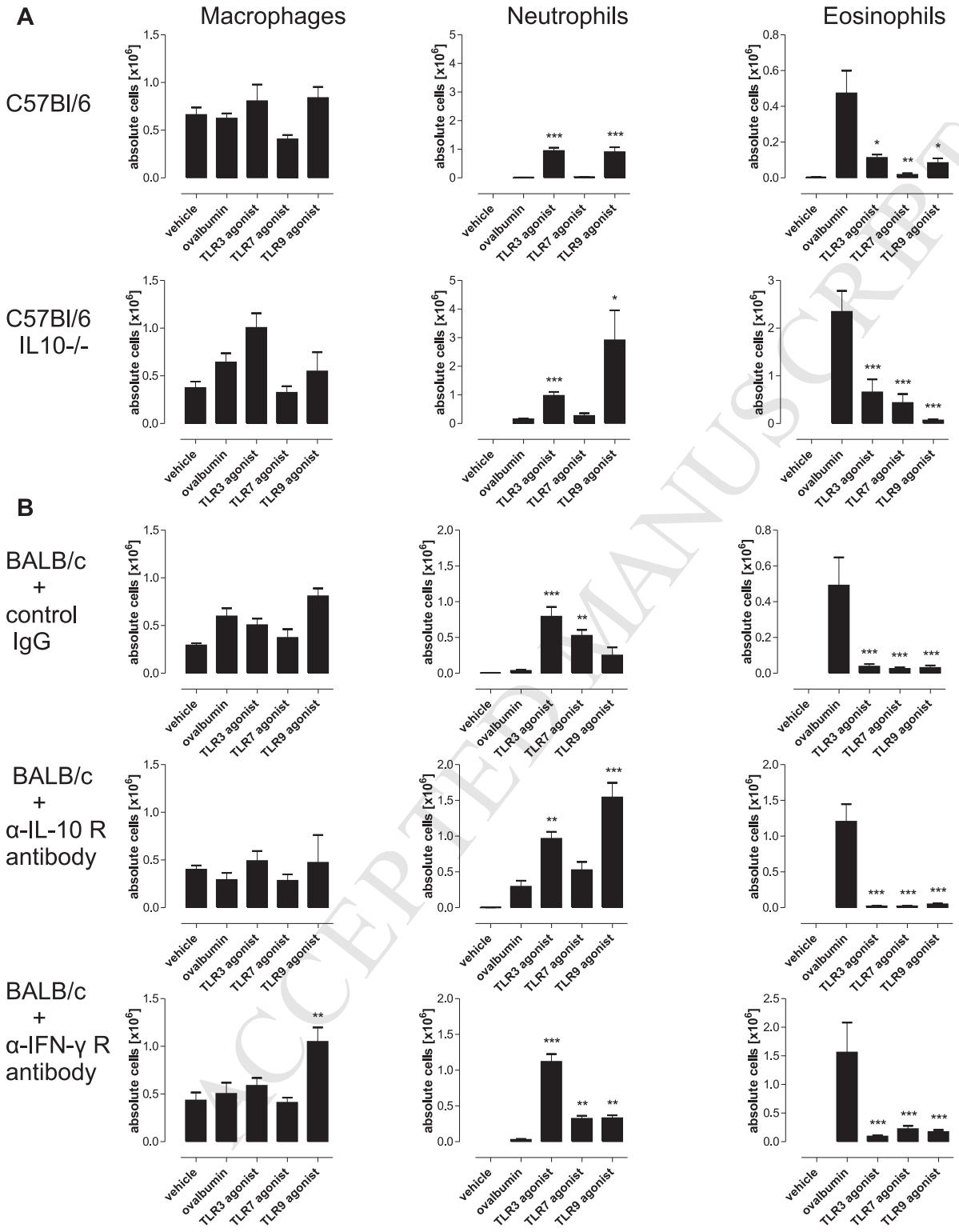

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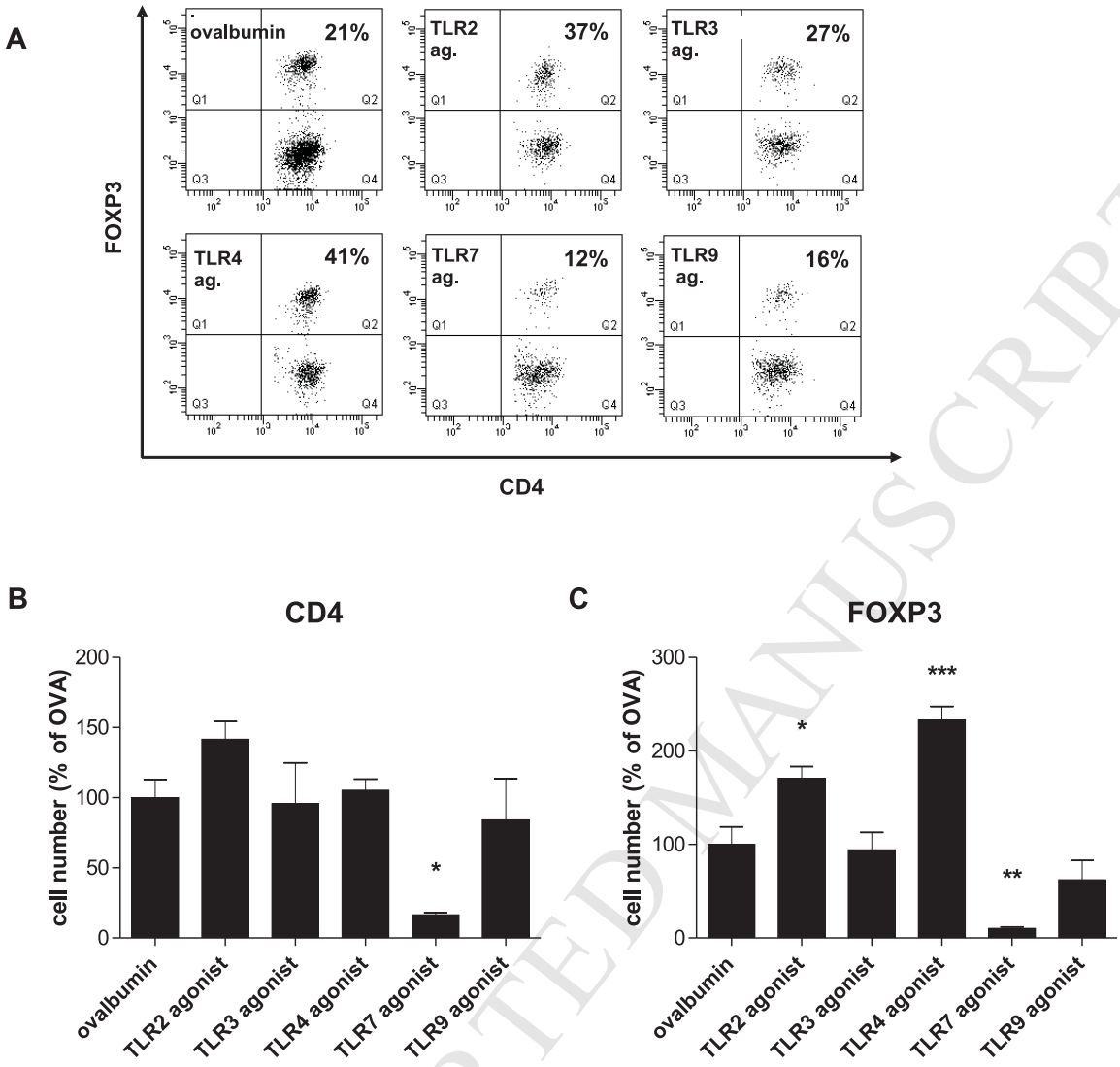

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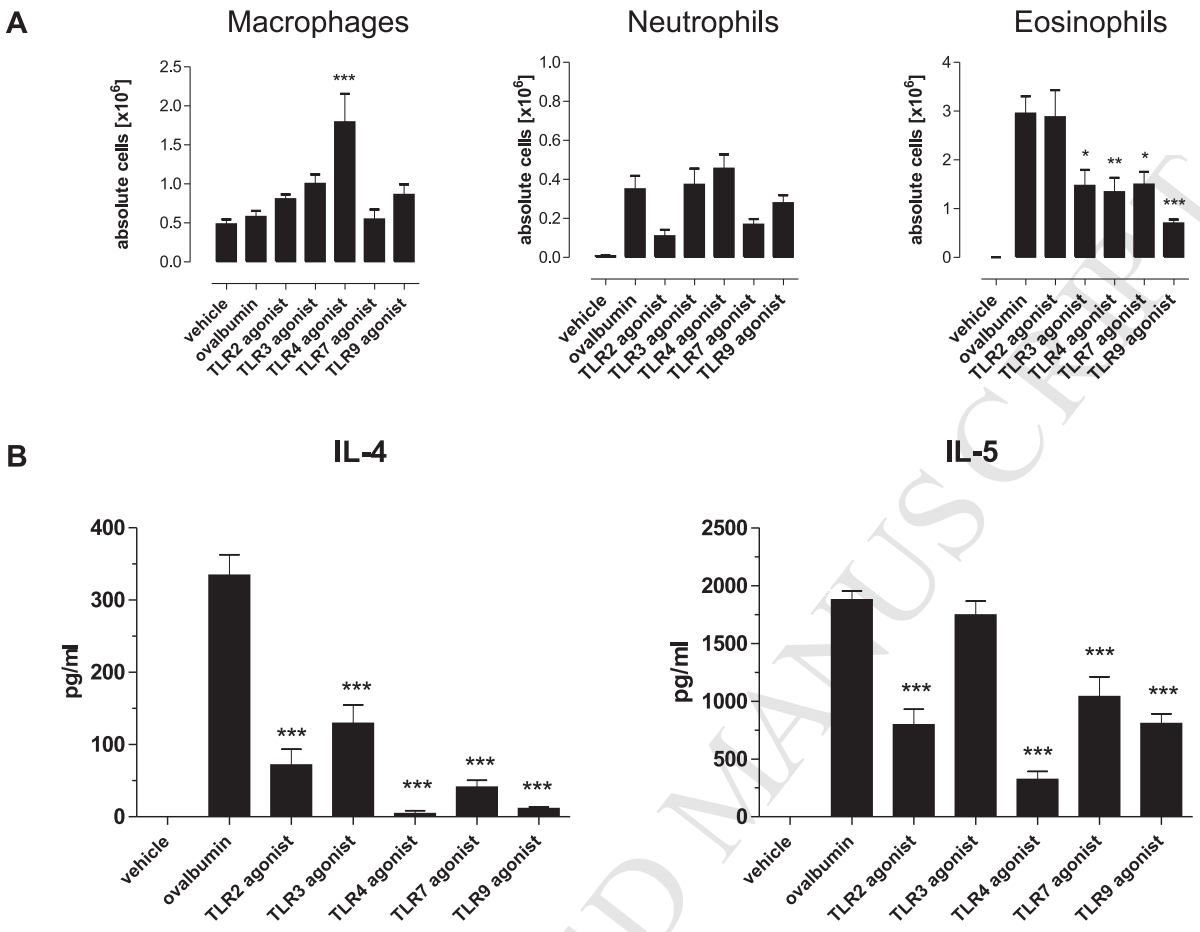
Figure 9



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