Thesis for Degree of Doctor of Philosophy (Ph.D)

Modulation of the humoral immune response by an FcγRII/III specific scFv in mice

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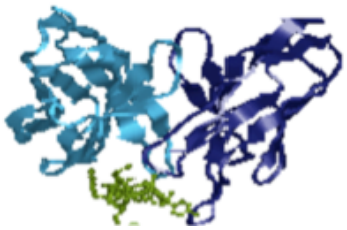

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Introduction

Fc-receptors play a central role in the regulation of both the efferent and afferent phase of the immune response by setting the threshold of B cell activation, regulating DC maturation and functioning as a bridge between innate and adaptive immunity. Due to the ability of immune complexes (IC) to bind all types of IgG Fc receptors via the conserved Fc-portion of IgG, activating type Fcγ-receptors (FcγR) may facilitate recruitment and activation of inflammatory cells and antigen presentation to effector cells, while the inhibitory FcγRIIb may transmit antagonistic signals with a possible outcome of inhibiting cell activation. Several *in vivo* studies aimed to examine the nature of this fine balance between positive and negative signals with yet controversial results.

Affinities of FcγRs are variable, low affinity receptors, FcγRII and FcγRIII only bind multiple Fc in immune complexes, and are less restricted to the isotype of the antibody, whilst the high affinity FcγRI shows a strict preference towards binding monomeric or complexed IgG2a.

Several groups reported on the *in vivo* immuno-modulatory effects of immune-complexes. IgG1-, IgG2a- and IgG2b-containing complexes can mediate immune response with dual outcomes, depending on the type of the antigen. Based on its unique structure, IgG3 has the capacity to self-associate and activate the complement system, and enhance the immune response. As IgG-mediated suppression also occurs in mice lacking the known FcγRs, this process is most possibly Fc-receptor independent, and caused by epitope masking. On the other hand, FcγRIIb-deficient mice are highly susceptible to a more severe form of collagen-induced arthritis, whilst animals lacking the common γ-chain of the activating Fc-receptors including FcγRI and FcγRIII do not develop the disease at all.

As the *in vivo* data about the regulatory function of immune complexes on the humoral immune response are controversial, we carried out a set of experiments where we aimed to collect more data about the localisation and *in vivo* effect of intravenously administered and well-characterised immune complexes. Several animal models of inflammatory diseases underline the importance of FcγR-function

therefore the further understanding of the complexity of FcγR-mediated signalling events could highly contribute to the development of efficient drugs diminishing inflammation in autoimmune diseases.

Aims of the study

1. Based on their structure, single chain Fragment variables (scFv) are a useful tool for cellular targeting experiments. During this study, first we aimed to produce and *in vitro* characterise a single chain Fragment variable (scFv) molecule from the well-described and widely used mouse CD16/32 specific clone 2.4G2.
2. While the antigen recognition features of the scFv are not supposed to be altered compared to the whole antibody, genetic modification and structural changes may disrupt antigen binding. Therefore, we wanted to examine the binding of the recombinant scFv to CD16/32 expressing target cells *in vitro* and *in vivo,* following intravenous injection. In order to establish a model immune complex, we biotinylated the 2.4G2 scFv enzymatically and mixed it with avidin-FITC, using the fluorescent dye as a hapten. Depending on the carrier molecule, FITC can induce T-cell-dependent and –independent responses, moreover, the dye can usually be detected hours after injection.
3. We aimed to analyse the effect of these immune complexes on the T-independent and T-dependent humoral immune responses. Therefore, we immunised mice with FITC-dextran (TI-2 antigen) or KLH-FITC (TD-antigen) and treated the animals with the model complex in different time points and combinations.
4. We wanted to study whether it is possible to target B cells via FcγRII by using these immune complexes and inhibit their activation (potential therapeutic importance in systemic autoimmune disorders).

Applied methods

- quantitative PCR, cloning and bioinformatical analysis of the sequences
- purification and refolding of recombinant proteins

- flow cytometry
- enzyme linked immunospot assay (ELISPOT)
- fluorescent microscopy
- SDS-polyacryl-amid gelelectrophoresis, dot blot

Results

In our experiments, we aimed to further investigate the regulatory function of ICs on the humoral immune response. To obtain well-defined ICs, from the antibody 2.4G2 we designed a biotinylated mouse FcγRII/III-specific scFv construct, coupled it to FITC-labelled avidin and used these model complexes to examine their regulatory role on FITC-specific TI-2 and TD immune responses.

First, we characterised the binding capacity of the pre-formed complex to spleen suspension cells *in vitro*. **Our assays showed that the antigen recognition of 2.4G2 scFv was intact, and comparable with the original antibody.** The *in vivo* experiments for binding assays demonstrated that the 2.4G2scFv-avidin FITC complex is present on a fraction of B220+, CD11b+ and CD11c+ splenocytes 15 and 60 minutes after intravenous injection, as shown by flow cytometry. However, unlike the mouse CR1/2 specific 7G6 scFv, the 2.4G2 scFv is filtered out from the circulation within 3 hours.

To track the localisation of the complexes within the spleen, we used immunofluorescent staining on frozen sections. **The intravenously injected model immune complexes were first partially localised on IBL9/2$^+$ sinusoid endothel cells then on MARCO$^+$ marginal zone macrophages, and were internalised 60 minutes post injection.** By the third hour of observation, neither the FITC signal nor the c-myc tag could be detected in the spleen.

Most surprisingly, despite of the relatively high expressions of FcγRII on marginal zone B cells, and our flow cytometric data, the complex did not stain the marginal zone B cells nor seemed to get through to the follicles within the white pulp of the spleen, probably due to the activity of marginal zone macrophages. The discrepancies between the data measured by flow cytometry and immunohistochemistry could

originate from differences in the sensitivity of the two detection methods, a phenomenon observed by other authors as well.

After having characterised the complex, we investigated its effect on T-independent and T-dependent B cell responses. We immunised mice with the TI-2 antigen FITC-dextran, and treated them with the complexes 36, 72 or 108 hours after antigen challenge. The targeted transport of the antigen to the marginal zone macrophages 108 hours following injection resulted in an elevated FITC-specific humoral response 6 days after primary immunisation.

As the scFv had been produced in a bacterial protein expression system, we had to rule out the possibility that the detected effect is mediated via bacterial lipopolysaccharid (LPS) and occurs independently from any FcγRII/III related events. By using a control scFv construct, derived from the mouse CR1/2 specific clone 7G6, we could not observe any significant changes in the number of FITC-specific antibody producing cells, nor could we by injecting LPS contaminated avidin-FITC to the control group. The addition of LPS-neutralising polymyxin B did not change the immune response enhancing effect of the complexes either, nor did the site of booster injection. In course of immunisation with the TD antigen KLH-FITC in complete Freund's adjuvant, the model ICs, b-2.4G2scFv-avidin-FITC significantly enhanced both IgM and IgG production to TD antigen as compared to the untreated control

Our results show that the enhanced IgM and IgG secretion in response to TI-2 and TD-antigens induced by the model immune complexes highly depends on FcγRII/III.

The monoclonal antibody 2.4G2 recognises both the activating CD16 and the inhibitory CD32 receptors, as these proteins show about 90 % sequence homology in their extracellular parts in mice. In order to decide which receptor is responsible for the enhanced antibody synthesis, we immunised CD16 knock out animals with both TI-2 and TD antigen, respectively, and compared their antibody production and its regulation by model immune complexes with the wild type littermates. CD16KO mice immunised with TI-2 antigen, dextran-FITC possessed of relatively higher numbers of hapten specific IgM producing cells in the spleen, so that the booster

injection with 2.4G2scFv-FITC complexes did not induce significant excessive B cell activation. We could not detect significant differences in the number of IgG producing cells either.

The antibody response to the TD antigen KLH-FITC was also highly significantly enhanced by 2.4G2scFv-avidin FITC complexes in case of wild type animals, while it showed a reduction in CD16KO mice, indicating that **FcγRIII is indispensable for the immune complex-induced enhancement of antibody synthesis**. The results suggest that immune complexes predominantly act *in vivo* as positive regulators of antibody production, which may be mediated by induction of cytokine synthesis. Immune complexes bound to FcγRIII on marginal zone macrophages may also promote antigen presentation to T cells or B cells, thus enhancing antibody synthesis.

From all these results we can conclude that the observed immunomodulatory effect of the 2.4G2 scFv- avidin FITC model IC is due to its binding to the FcγRIII, most probably on the marginal zone macrophages.

The thesis is based on the following publications:

Angyal A., Szekeres Zs., Balogh P., Neer Zs., Szarka E., Virag V., Medgyesi D., Prechl J., Sarmay G.: CD16/32 specific biotinylated 2.4G2 single chain Fv complexed with avidin-FITC enhances FITC-specific humoral immune response in vivo in a CD16-dependent manner (accepted for publication in Int. Immunol) (IF: 4.015)

Angyal A, Prechl J, Sarmay G.: Possible therapeutic applications of single-chain antibodies in systemic autoimmune diseases. Expert Opin Biol Ther. 2007 May;7(5):691-704. (IF: 2.705)

Other publications:

Eder K, Guan H, Sung HY, Ward J, Angyal A, Janas M, Sarmay G, Duda E, Turner M, Dower SK, Francis SE, Crossman DC, Kiss-Toth E.: Tribbles-2 is a novel regulator of inflammatory activation of monocytes. Int. Immunol. 2008 Dec. 20(12):1543-50 (IF: 4.015)

Angyal A, Medgyesi D, Sarmay G.: Grb2-associated binder 1 (Gab1) adaptor/scaffolding protein regulates Erk signal in human B cells.Ann N Y Acad Sci. 2006 Dec;1090:326-31. (IF: 1.930)

Sarmay G, Angyal A, Kertesz A, Maus M, Medgyesi D.: The multiple function of Grb2 associated binder (Gab) adaptor/scaffolding protein in immune cell signaling. Immunol Lett. 2006 Apr 15;104(1-2):76-82. Epub 2005 Dec 13. (IF:2.352)

Sarmay G, Kertesz A, Takacs B, Angyal A, Medgyesi D, Váradi G, and Tóth G. The role of Grb2 associated binder 1 (Gab1) scaffolding adaptor protein on signal transduction in human B cells. Febs Journal 272: 323-324 Suppl. 1 July 2005 (IF: 3.033)