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Anti-CD25 monoclonal Antibody (mAb): an immunomodulating drug candidate for the treatment of T-Cell mediated diseases

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Index

Table of Figures

Table of Tables

1. INTRODUCTION and BACKGROUND

IL-2 is a potent immunomodulator whose major function is the activation of various cells of the immune system including T cells, B cells, NK cells and macrophages.
Its receptor is expressed at low levels on around 5% of resting peripheral blood mononuclear cells but is rapidly transcribed and expressed upon cell activation and is implicated in the generation of effector cells from antigen stimulated T cells.
Interestingly it has been shown to be expressed constitutively by a small population of regulatory T cells, which have a suppressive activity.

Given the potent immunomodulatory effect of IL-2 on T cells and the fact that the IL-2 receptor (in particular IL-2Rα also known as CD25 antigen) has been found to be highly expressed by T cells in some autoimmune diseases, a monoclonal antibody designed to bind with high affinity the CD25 antigen has the potential to prevent at least some of the biological activities of IL-2.
However, it is difficult to foresee which sub population of CD25+ cells will be most inhibited by anti-CD25 blocking antibodies in vivo and such differential effects would impact the potential clinical use since the key to successful anti- CD25 therapy is to elicit the correct balance between effector and regulatory T cells.

The therapeutic antibody presented herein designated as *Anti- CD25 mAb* is a fully human $IgG1_k$ monoclonal antibody (mAb) against IL-2Rα generated using transgenic mice containing an unrearranged human antibody repertoire in which endogenous murine antibody expression has been knocked out. Mice were then immunized with soluble and cell-bound CD25. Hybridoma cell cultures originating from the fusion of murine splenocytes and a human myeloma cell line were then established for the production of anti-CD25 monoclonal antibodies.
Among the different monoclonals obtained, the drug candidate antibody was selected based on its ability to inhibit the binding of IL-2 to IL-2Rα in comparison to two commercially available products (a humanized anti- IL-2R and a chimeric human and mouse anti- IL-2R).
The potential competitive advantages of *Anti- CD25 mAb* are 3-5 times higher affinity for human CD25 than competitors and the fact that it is a fully humanised mAb, potentially less immunogenic, leading to longer duration of therapeutic effect.

A number of potential therapeutic indications were then envisaged for *Anti- CD25 mAb* including rheumatoid arthritis (RA), multiple sclerosis (MS), scleroderma, skin disorders like psoriasis and atopic dermatitis, ulcerative colitis, asthma, type 1 diabetes and heart transplant rejection. Among those, it is felt that the great therapeutic promise could in the clinical setting of different forms of MS.
Hence, *Anti- CD25 mAb* is presented with a potential therapeutic application in T cell mediated diseases and with primary indication of Multiple Sclerosis (MS).

1.1 CD25 (Interleukin-2 receptor) and autoimmune diseases

1.1.1 CD25 / TAC: α-chain of IL-2 receptor

CD25 / TAC is the unique α-chain of the IL-2 receptor (IL-2Rα).

IL-2 is a key regulator of normal immune functions, acting on T cells, B cells and natural killer cells. CD25 is highly expressed on activated T cells and is a clinically validated target for the development of novel mAb therapies that block IL-2R function inhibiting, in turn, T cell proliferation.

CD25-specific mAb therapies have proven efficacy in the treatment of a number of T cell mediated diseases, including organ transplant rejection and autoimmune disease, such as multiple sclerosis (MS) and uveitis.

1.1.1.1 Structure and function of the IL-2 receptor

The high-affinity interleukin-2 receptor (IL-2R) consists of three subunits: the α-chain (IL-2Rα; also known as CD25), the β-chain (IL-2R β; also known as CD122) and the common cytokine-receptor γ-chain (γ c; also known as CD132). It is thought that in the absence of IL-2, these subunits are not pre-assembled on the surface of an IL-2R-expressing T cell. The concomitant expression of the all three chains is indispensable for IL-2 signaling to be executed [1].

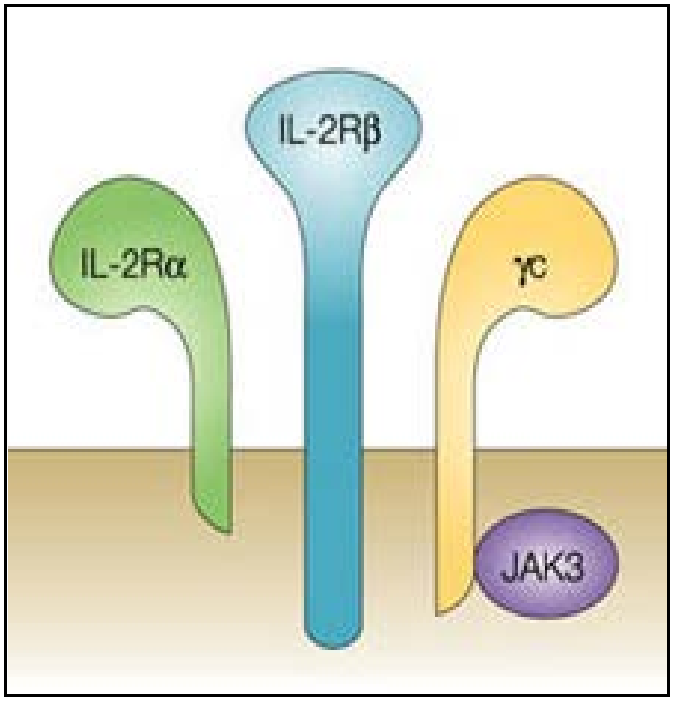


Fig. 1: *IL-2 receptor (IL-2R) subunits: α- (CD25), β- (CD122) and γ-chains (CD132)*

The binding of IL-2 to IL-2Rα drives the association of this subunit with IL-2R β and the γ chain to form a stable heterotrimer, which then leads to the initiation of signal transduction.

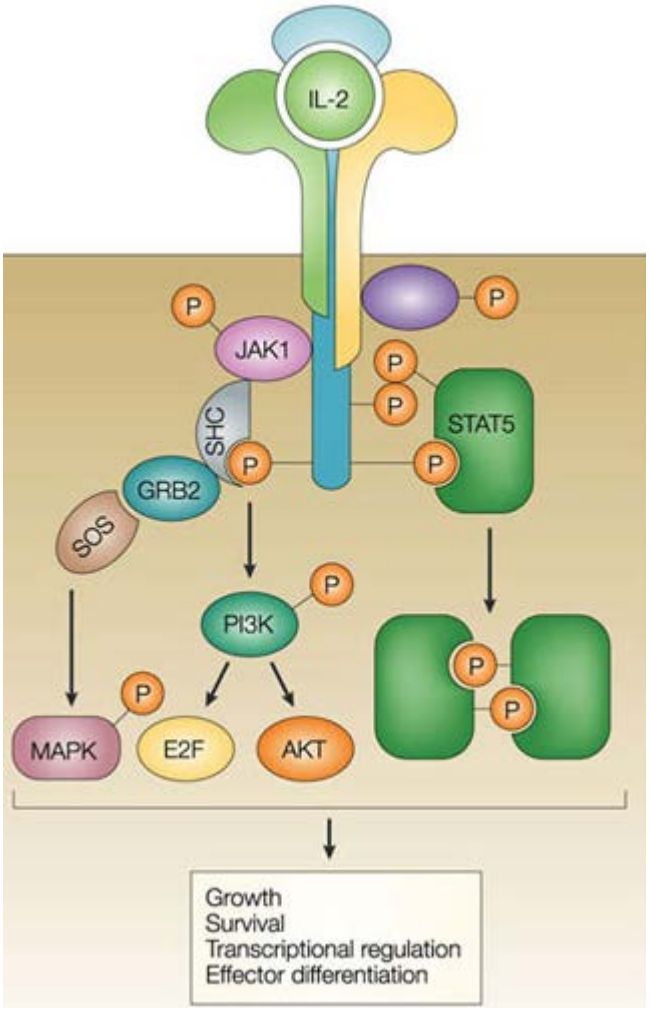


Fig. 2: *IL-2 activation pathway*

Janus activated kinase 3 (JAK3) molecules that are associated with the ᵞ c, and JAK1 molecules that are associated with IL-2R β, phosphorylate key tyrosine residues in the cytoplasmic tail of IL-2R β, the ᵞ c and the JAK molecules themselves. This amplifies the association of these tyrosine kinases and induces the association of the adaptor SHC (SRC-homology-2-domain-containing transforming protein C), and either signal transducer and activator of transcription 5 (STAT5) or STAT3, with the cytoplasmic tail of IL-2R β. The complexed SHC allows activation of the mitogen-activated protein kinase (MAPK)- and phosphatidylinositol 3-kinase (PI3K)–AKT-signalling pathways. Tyrosine residues in STAT5 are also phosphorylated, leading to the dimerization of STAT5 and its translocation to the nucleus where it regulates STAT5-responsive genes. GRB2, growth-factor-receptor-bound protein 2; SOS, son of sevenless homologue.

The common γ-chain is shared by the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 and its mutation causes severe combined immunodeficiencies [2]. The β - and γ chain without the α-subunit build the β -/γ- complex, a form of the IL-2R with intermediate affinity, which also binds IL-15. Therefore, both cytokines (IL-2 and IL-15) compete for binding to the β -/γ- complex. This intermediate affinity IL-2R is present on NK cells and resting T cells [3]. Different α-chains for the IL-2 receptor, together with the β -/γ- complex, complete the high-affinity form of each receptor [4]. Upon activation, expression of the α-chain (CD25) is rapidly up-regulated on the cell surface [5]. Activated T cells, regulatory T cells (T_{reg}s) [1] and NK cells [6] express high levels of CD25 and expression of the high-affinity IL-2R is mostly limited to these cell populations. Isolated expression of the α-chain (CD25) is not sufficient to trigger the IL-2 signaling pathway because CD25 by itself displays the low-affinity form of the IL-2R that is non-functional with respect to IL-2 internalization and signaling due to its short cytoplasmic tail [5]. CD25 is mainly involved in IL-2 binding thereby stabilizing a secondary binding site for the

β-chain and recruiting γ-chain to the cell surface [7]. Despite the absolute requirement for the γ-chain in IL-2 signaling, the signal transduction itself does not act via its cytoplasmic tail. The majority of known pathways are linked to the receptor via the β -chain, generally through phosphorylated cytoplasmic tyrosine residues that recruit various non-receptor-type protein tyrosine kinases (PTKs), which trigger an intracellular signaling cascade [8]. Furthermore, it has been demonstrated that the IL-2R complex is linked to at least three distinct signaling pathways leading to the induction of the target genes *c-fos/c-jun*, *c-myc* and *bcl-2* [9]. All these pathways are essential for IL-2- mediated proliferative signaling and co-operate with each other to ensure a full-scale signal transduction [8].

In contrast to β- and γ-chains, CD25 also exists in a soluble form that not the product of a unique post-transcriptional splicing event [10] but is most likely derives from proteolytic cleavage of surface CD25 [11] rather than originating from a unique post-transcriptional splicing event [10]. The soluble form of CD25 binds IL-2 efficiently [12] but with lower affinity than the β-/γ complex, with a dissociation constant similar to that of surface CD25 [13].

Signaling via IL-2R mediates multiple biological processes in various cell populations, e.g. proliferation and differentiation of B cells [14] and NK cells [15]. However, T cells remain the major target of IL-2 [16]. IL-2 is secreted after encounter of the TCR/MHC-complex (the antigen presenting cell and T cell receptor interaction is required for antigen recognition by T lymphocytes), binds autocrine to the high affinity IL-2R on the T-cell surface and thereby allows entrance into the cell cycle and clonal expansion of activated T cells [3]. IL-2 is produced primarily by activated CD4+ T cells and CD8+ T cells [17].

Interestingly, involvement of IL-2 signaling in the homeostasis of CD25high T_{reg}s has been described [1]. T_{reg} cells are a minor subpopulation of T cells that develop in the thymus and migrate to the periphery, where their main role is to suppress self-reactive T cells that escape negative selection in the thymus. T_{reg} cells not only prevent autoimmunity but also control a broad range of T-cell-dependent immune responses *in vivo* [18]. So, functionally T_{reg} cells also inhibit the rejection of transplants, prevent the induction of antitumour responses and regulate the immune response to infectious diseases. Although much remains to be learned about the basis of T_{reg} -cell generation, specificity and inhibitory activity, there is increasing evidence that points to a mandatory role for IL-2–IL-2R signalling in the development and peripheral activity of T_{reg} cells [19].

1.1.1.2 IL -2 and T –Cell response

The idea that IL-2 is an essential cytokine for the production of T_{reg} cells is based on recent data. However, the importance of IL-2 as a potent T-cell growth factor has been recognized for more than 20 years.
Three key findings, which have been well-documented in in-vitro studies, led to a widely held view that IL-2 is an essential cytokine for both generating and then limiting T-cell-dependent immune responses [20].

First, IL-2 has potent T-cell growth-factor activity [21]. The genes that encode IL-2 and the subunits of the IL-2R, especially IL-2Rα, are among the immediate early genes that are activated in T cells as a consequence of signalling through the TCR. After either CD4+ or CD8+ T cells have been activated, exogenous IL-2 levels are sufficient to induce a more than 1000-fold clonal expansion of these cells. Other cytokines have also been reported to function as T-cell growth factors, notably those with receptors that contain the γc [22].

However, IL-2 is the most potent T-cell growth factor and the main γc-dependent cytokine that is secreted when T cells are initially activated *in vitro*.

Second, T-cell proliferation and function *in vitro* can be substantially inhibited using monoclonal antibodies specific for either IL-2 or the IL-2R [23]. This finding provides the strongest support for a crucial role for IL-2 in the generation of an immune response. Furthermore, the ability to block many such responses supports the view that TCR ligation *(signal 1)* and co-stimulatory-receptor(s) engagement *(signal 2)* induces the production of IL-2 and the expression of the IL-2R; the subsequent IL-2–IL-2R interaction is then responsible for the proliferation of antigen-activated T cells. Indeed, when the *precursor frequency* of the responding T cells is relatively low, such as for cells specific for antigen or alloantigen, blockade of the IL-2–IL-2R interaction abrogates T-cell proliferative responses.

However, after strong stimulation through the TCR, such as that induced by mitogens or CD3-specific antibody, T-cell proliferation is sometimes only partially inhibited by IL-2- or IL-2R-specific antibodies [24].
It was argued that the failure to block these responses resulted from limitations of the inhibitory activity of the monoclonal antibodies. To block these responses, IL-2-specific or IL-2R-specific antibodies must substantially inhibit most of the IL-2–IL-2R interaction for a considerable time, under conditions in which the affinity of IL-2 for its receptor is ~2–3 orders of magnitude higher than the monoclonal antibody binding to these proteins. Nevertheless, the high concentration and potency of the antibodies used were such that more substantial inhibition of proliferation would be anticipated if proliferation was driven by IL-2. More-recent data clearly indicate that some T-cell proliferation occurs *in vitro* in an IL-2R-independent manner. This has best been shown using IL-2- or IL-2R-deficient mice, in which substantial proliferation to mitogen, CD3-specific antibody or specific antigen has been reported — although these responses were always reduced compared with those of control mice. The ability of T cells that are deficient in the IL-2–IL-2R pathway to respond to stimulation was not the result of an abnormal T-cell response occurring as a consequence of autoimmunity, because T-cell proliferation of IL-2- or IL-2R-deficient T cells occurred after the prevention of autoimmunity in these mice. Prevention of autoimmunity can be accomplished either by breeding TCR-transgenic mice onto IL-2-/- or IL-2Rα -/- genetic backgrounds so that the mice only express TCRs of a single defined specificity and therefore do not have autoreactive T cells, or it can be achieved by providing T_{reg} cells to such mice. It is important to note that when IL-2- or IL-2R-deficient T cells from mice that are free from autoimmunity are activated to proliferate *in vitro*, these T cells are markedly deficient in effector functions, such as cytokine secretion and cytotoxic T lymphocyte (CTL) activity [25].

This finding indicates that a failure in effector function is not due to a lack of T-cell growth. Therefore, the simplest interpretation of these findings is that engagement of the TCR and co-stimulatory molecules is sufficient to induce several rounds of T-cell proliferation *in vitro*, but more-extensive clonal expansion and differentiation into effector cells depend on IL-2. The molecular basis by which IL-2 controls effector-cell differentiation has not been extensively investigated, but recent studies indicate that IL-2 stabilizes the accessibility of the IL-4 gene, thereby influencing IL-4 secretion [26].

The ***third*** key activity of IL-2, which was also determined using *in vitro* studies, is its ability to sensitize activated T cells to undergo apoptosis or activation-induced cell death (AICD) by FAS- and tumour-necrosis factor (TNF)-dependent pathways [27]. AICD occurs when the TCR is engaged by antigen after substantial clonal expansion in response to IL-2 *in vitro*. It is thought that AICD is an important mechanism that greatly reduces the number of antigen-specific T cells and limits the immune response. A failure in AICD of autoreactive T cells was postulated to

account for the lymphoproliferation and lethal autoimmunity that is associated with IL-2-deficiency and IL-2R-deficiency. Other γc-dependent cytokines, notably IL-4 and IL-7, also sensitize T cells to undergo AICD, but not as efficiently as IL-2 [28]. So, all of these *in vitro* studies place IL-2 in a central role for T-cell responses, by controlling the magnitude of clonal expansion, the development of effector cells and the subsequent contraction of the antigen-specific T cells.

1.1.1.3 IL -2 as a "signal 3" T –Cell immunity

On the basis of the above information, it was proposed a model to account for the distinct requirements for IL-2 during T-cell-dependent immune responses *in vitro* and *in vivo* (Figure 3 - [1]).

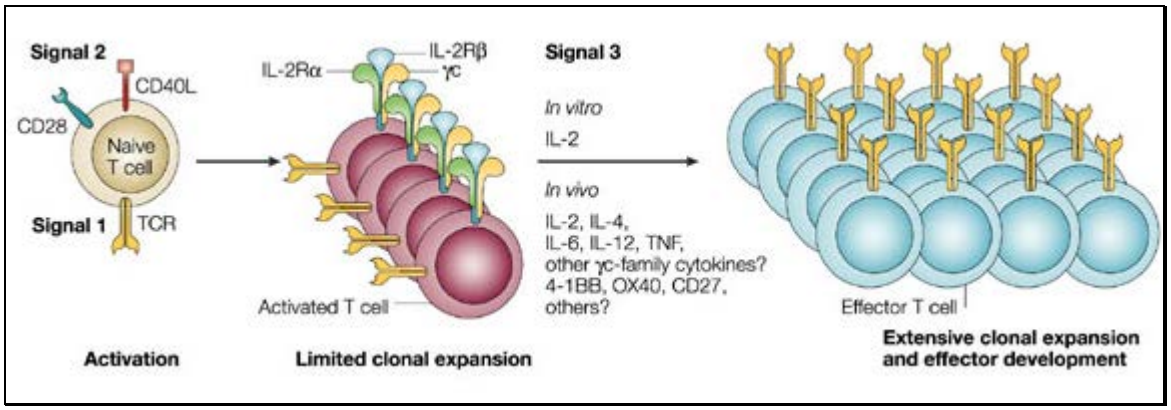


Fig. 3: *IL-2 as a "signal 3" T-Cell immunity*

A naive T cell is activated after ligation of its T-cell receptor (TCR) (signal 1) and engagement of the co-stimulatory molecules CD28 and CD40 ligand (CD40L) (signal 2) during antigen presentation by a dendritic cell (DC). This is sufficient to induce several rounds of T-cell proliferation; however, this interaction is not sufficient for an effective T-cell-dependent immune response. Signal 3 is a crucial checkpoint for substantial clonal expansion of antigen-specific T cells and development into effector cells. In tissue culture, engagement of the interleukin-2 receptor (IL-2R) is the main mechanism of passing the signal 3 checkpoint. However, there are several sources of signal 3 in vivo. It is probable that redundancy in the molecules that provide signal 3 is not limited to other common cytokine-receptor –γ chain (γc)-dependent cytokines.

Engagement of the TCR *(signal 1)* and co-stimulatory molecules *(signal 2)* is sufficient to induce limited clonal expansion of T cells *in vitro*, but extensive amplification of these T cells and their differentiation into effector cells requires signals through the IL-2R *(signal 3).* This activity of IL-2 might be viewed as a crucial checkpoint that is required for a productive T-cell response.

However, during *in vivo* T-cell-dependent immune responses in the absence of IL-2, considerable T-cell clonal expansion occurs, but effector-cell responses are impaired to a variable extent. Nevertheless, a protective immune response still ensues. In the absence of IL-2 *in vitro*, one prediction of this model is that providing additional signals should restore one or more aspects of impaired T-cell activity. Supplementing cultures of IL-2- or IL-2R-deficient T cells with exogenous IL-4, IL-6 or TNF improved T-cell growth and effector activity [29]. So, both inflammatory and T-cell-derived cytokines might provide *signal 3.* Several members of the TNF-receptor superfamily, such as 4-1BB (also known as CD137), OX40 (also known as CD134) and CD27, are induced during T-cell activation and strongly enhance T-cell responses. These might also be candidates for *signal 3 in vivo*. Regardless of which molecule(s) can bypass IL-2 *in vivo*, its activity is more effective in reconstituting T-cell proliferation than effector activity, because defects in effector function were more commonly observed in IL-2-deficient and IL-2R-deficient mice. This also indicates that *signal 3* is complex, because normal clonal

expansion of T cells is not always accompanied by a normal effector response. One simple explanation that could account for IL-2-independent T-cell clonal expansion *in vivo* is that other γ c-dependent cytokines provide this function. In some cases, this might be sufficient, because a JAK3-specific inhibitor has been shown to prolong survival of heart and kidney transplant [30].

However, when γc-deficient TCR-transgenic $CD4^+$ T cells are challenged with antigen, substantial clonal expansion of these T cells readily occurred, showing that T cells clonally expand in response to antigen *in vivo* in the absence of signalling induced by any of the six cytokines that depend on the γc as a receptor subunit [31].

1.1.1.4 Clinical implications

Mouse studies indicate that *in vivo* IL-2 is crucial for the differentiation of T_{reg} cells and therefore for the maintenance of peripheral T-cell tolerance that it is required for the generation of effective T-cell-mediated immunity. This concept should now be considered when targeting IL-2 or the IL-2R in clinical settings, because $CD4^+$ T_{reg} cells (highly expressing CD25) and a functional role for IL-2 have also been identified in humans [32] [33]. A mutation in the CD25 gene that leads to a lack of CD25 expression as well as of the high affinity IL-2R has been reported in humans. This patient had anaemia, lymphadenopathy and multi-organ inflammatory infiltrates [34], symptoms that are similar to those of mice that are deficient in IL-2 or the IL-2R. Given this information and our new appreciation for the important contribution of IL-2 to the production of T_{reg} cells, targeting IL-2 and/or the IL-2R in clinical settings is not synonymous with directly targeting the clonal expansion and development of effector T cells.

IL-2 is being used in clinically to boost immunity in individuals who are infected with HIV and patients with AIDS, and also in patients with cancer, especially those with metastatic melanoma, acute myelogenous leukaemia or metastatic renal-cell carcinoma [35] [36]. Although IL-2 might boost an effector response, it might be prudent to consider whether this might also increase the number or potency of T_{reg} cells. Such an effect could diminish immune responses, which would be undesirable if the goal of therapy is to augment immunity. The presence of T_{reg} cells is also crucial for the maintenance of homeostasis of both peripheral CD4+ and CD8+ T cells, partly by preventing unwanted autoantigen-dependent T-cell activation and by controlling CD8+ memory T cells [37]. So, the capacity of IL-2 to reset an imbalanced immune compartment, as observed in HIV/AIDS patients [38], might be attributed partly to restoring T_{reg} cells, rather than solely to boosting effector T cells. Consistent with this hypothesis, recent data show that treatment of HIV patients with IL-2 leads to decreased proliferation of CD4+ T cells while increasing the number of both naïve and memory T cells [39]. Interestingly, these changes were found to be associated with a clonal expansion of CD4+CD25+ T cells. However, it is still to be investigated if these cells are T_{reg} cells.

Investigational CD25-specific antibodies have been reported to transiently deplete peripheral T_{reg} cells in mice [40]. Another class of agent that is potently cytotoxic for IL-2R+ cells is IL-2–diphtheria toxoid fusion proteins, and these have been used clinically to target IL-2R+ lymphomas [41]; however, they could also target T_{reg} cells. More- broadly acting drugs that potentially antagonize the action of IL-2, such as rapamycin, or drugs that suppress IL-2 production, such as cyclosporin, might also affect T_{reg} cells. Such outcomes might result in unwanted augmentation of immunity or induction of an autoimmune response, rather than inhibition of the undesirable immune response.

By contrast, this inhibition of IL-2 or depletion of CD25+ T cells, which might reduce the

number and function of T_{reg} cells, could be beneficial for tumour immunity, because in this situation, the main goal is to increase self-reactivity towards tumour-associated antigens. Examples of enhanced tumour immunity have been reported in mouse models after depletion of T_{reg} cells [42], and increased numbers of T_{reg} cells have been detected in human cancer patients [43], raising the possibility that these cells might suppress the host immune response. However, to determine whether current interventions that target the IL-2R actually interfere with T_{reg} -cell activity needs to be evaluated in patients undergoing therapy with CD25-specific antibodies or other agents that target IL-2 production. Because this is a serious issue, it also necessitates a better assessment of whether IL-2 is essential for the maintenance and homeostasis of peripheral T_{reg} cells. In this regard, if the function of IL-2 is crucial only during thymic development, then redundant pathways might be sufficient to maintain the number and function of peripheral T_{reg} cells, even when IL-2 is blocked in an immunocompetent individual. Consistent with this idea, it was reported that thymic reconstitution of the IL-2R was sufficient to correct the severe autoimmune syndrome that in mice is associated with IL-2Rβ -/- phenotype, even though the resulting peripheral T_{reg} - cell pool does not express a functional IL-2R [44]. However, more studies are required to establish the contribution of IL-2 to peripheral mouse and human T_{reg} cells.

In clinical settings, IL-2R blockade has shown some efficacy in graft vs host disease, including transplant rejection and autoimmune diseases [45] [46]. The mechanism by which IL-2R-specific antibodies suppress these responses has not been precisely established, but these antibodies are thought to inhibit the activity of IL-2 on activated T cells. It might be relevant that CD25-specific monoclonal antibodies are often used in combination with, or directly after, other immunosuppressant that more-broadly target T-cell activation. To achieve a clinically relevant inhibitory response, the utility of targeting the IL-2R together with other aspects of T-cell activation is consistent with the observation, in mouse models, of an operational redundancy for the effective T-cell immune responses even when IL-2R function is absent.
There is now evidence that the impaired production of T_{reg} cells is sufficient to account for the lethal autoimmunity that is associated with IL-2-, IL-2Rα- and IL-2Rβ – deficiency in mice. Therefore, the main function of IL-2 seems to be the production of T_{reg} cells and the maintenance of peripheral T-cell tolerance. One of the most important unanswered questions is what the outcomes of signalling through the IL-2R are for the development, homeostasis and functional activity of T_{reg} cells. Furthermore, in therapeutic settings, manipulation of T_{reg} -cell number or function might be accomplished by targeting IL-2 or the IL-2R. Conceptually, this is counter-intuitive to the past emphasis on IL-2 as a driver of immune responses. But consistent with this hypothesis, challenge of IL-2- or IL-2R-deficient mice that have been rendered free of autoimmunity clearly shows that effective T-cell immunity is generated in the absence of IL-2. Therefore, although IL-2 is a dominant signal 3 for the generation of T-cell responses *in vitro*, there must be sufficient redundancy *in vivo* to compensate for the lack of IL-2. So, enhanced understanding of the nature of signal 3 in vivo, at both the immunological and molecular levels, might lead to better defined targets that can be used to inhibit both T-cell clonal expansion and effector responses.

1.2 The Guideline ICH S6 (R1): Preclinical Safety Evaluation of Biotechnology – Derived Pharmaceuticals

The ICH S6 guidance has been issued to provide general principles for designing scientifically acceptable preclinical safety evaluation programs, and may offer useful background in developing new biopharmaceuticals.

The preclinical safety evaluation is needed to support clinical development and marketing authorization.

The primary goals of preclinical safety evaluation are:

1. to identify an initial safe dose and subsequent dose escalation schemes in humans;
2. to identify potential target organs for toxicity and for the study of whether such toxicity is reversible;
3. to identify safety parameters for clinical monitoring.

This guidance is intended primarily to recommend a basic framework for the preclinical safety evaluation of biotechnology-derived pharmaceuticals. It applies to products derived from characterized cells through the use of a variety of expression systems including bacteria, yeast, insect, plant, and mammalian cells. The intended indications may include *in vivo* diagnostic, therapeutic, or prophylactic uses. The active substances include proteins and peptides, their derivatives, and products of which they are components; they could be derived from cell cultures or produced using recombinant deoxyribonucleic acid (DNA) technology, including production by transgenic plants and animals. Examples include but are not limited to: Cytokines, plasminogen activators, recombinant plasma factors, growth factors, fusion proteins, enzymes, receptors, hormones, and monoclonal antibodies.

The principles outlined in this guidance may also be applicable to recombinant DNA protein vaccines, chemically synthesized peptides, plasma derived products, endogenous proteins extracted from human tissue, and oligonucleotide drugs.

This document does not cover antibiotics, allergenic extracts, heparin, vitamins, cellular blood components, conventional bacterial or viral vaccines, DNA vaccines, or cellular and gene therapies.

1.2.1 Specification of the Test Material

Safety concerns may arise from the presence of impurities or contaminants.
It is preferable to rely on purification processes to remove impurities and contaminants rather than to establish a preclinical testing program for their qualification. In all cases, the product should be sufficiently characterized to allow an appropriate design of preclinical safety studies.

There are potential risks associated with host cell contaminants derived from bacteria, yeast, insect, plants, and mammalian cells. The presence of cellular host contaminants can result in allergic reactions and other immunopathological effects. The adverse effects associated with nucleic acid contaminants are theoretical but include potential integration into the host genome. For products derived from insect, plant, and mammalian cells, or transgenic plants and animals,

there may be an additional risk of viral infections.

In general, the product that is used in the definitive pharmacology and toxicology studies should be comparable to the product proposed for the initial clinical studies.

The comparability of the test material during a development program should be demonstrated when a new or modified manufacturing process is developed or other significant changes in the product or formulation are made in an ongoing development program. Comparability can be evaluated on the basis of biochemical and biological characterization (i.e., identity, purity, stability, and potency). In some cases, additional studies may be needed (i.e., pharmacokinetics, pharmacodynamics and/or safety). The scientific rationale for the approach taken should be provided.

1.2.2 Preclinical Safety Testing

The objectives of the preclinical safety studies are to define pharmacological and toxicological effects not only prior to initiation of human studies but throughout clinical development. Both *in vitro* and *in vivo* studies can contribute to this characterization. Biopharmaceuticals that are structurally and pharmacologically comparable to a product for which there is wide experience in clinical practice may need less extensive toxicity testing.

Preclinical safety testing should consider:

1. Selection of the relevant animal species;
2. age;
3. physiological state;
4. the manner of delivery, including dose, route of administration, and treatment regimen;
5. stability of the test material under the conditions of use.

Toxicity studies are expected to be performed in compliance with Good Laboratory Practice(GLP); however, it is recognized that some studies employing specialized test systems, which are often needed for biopharmaceuticals, may not be able to comply fully with GLP. In some cases, lack of full GLP compliance does not necessarily mean that the data from these studies cannot be used to support clinical trials and marketing authorizations.

Conventional approaches to toxicity testing of pharmaceuticals may not be appropriate for biopharmaceuticals due to the unique and diverse structural and biological properties of the latter that may include species specificity, immunogenicity, and unpredicted pleiotropic activities.

1.2.2.1 Biological activity / Pharmacodynamics

Biological activity may be evaluated using *in vitro* assays to determine which effects of the product may be related to clinical activity. Due to the species specificity of many biotechnology-derived pharmaceuticals, it is important to select relevant animal species for toxicity testing.
In vitro cell lines derived from mammalian cells can be used to predict specific aspects of *in vivo* activity and to assess quantitatively the relative sensitivity of various species (including human) to the biopharmaceutical. The combined results from *in vitro* and *in vivo* studies assist in the extrapolation of the findings to humans. *In vivo* studies to assess pharmacological activity, including defining mechanism(s) of action, are often used to support the rationale of the proposed use of the product in clinical studies.

For monoclonal antibodies, the immunological properties of the antibody should be described in detail, including its antigenic specificity, complement binding, and any unintentional reactivity and/or cytotoxicity towards human tissues distinct from the intended target. Such cross-reactivity studies should be carried out by appropriate immunohistochemical procedures using a range of human tissues.

1.2.2.2 Animal species / Model selection

The biological activity together with species and/or tissue specificity of many biotechnology derived pharmaceuticals often preclude standard toxicity testing designs in commonly used species (e.g., rats and dogs). Safety evaluation programs should include the use of relevant species. A relevant species is one in which the test material is pharmacologically active due to the expression of the receptor or an epitope (in the case of monoclonal antibodies). Knowledge of receptor/epitope distribution can provide greater understanding of potential *in vivo* toxicity.

Relevant animal species for testing of monoclonal antibodies are those that express the desired epitope and demonstrate a similar tissue cross-reactivity profile as for human tissues. This would optimize the ability to evaluate toxicity arising from the binding to the epitope and any unintentional tissue cross-reactivity.

Safety evaluation programs should normally include two relevant species. However, in certain justified cases one relevant species may suffice (e.g., when only one relevant species can be identified or where the biological activity of the biopharmaceutical is well understood).

Toxicity studies in non-relevant species may be misleading and are discouraged. When no relevant species exists, the use of relevant transgenic animals expressing the human receptor or the use of homologous proteins should be considered. While useful information may also be gained from the use of homologous proteins, it should be noted that the production process, range of impurities/contaminants, pharmacokinetics, and exact pharmacological mechanism(s) may differ between the homologous form and the product intended for clinical use. Where it is not possible to use transgenic animal models or homologous proteins, it may still be prudent to assess some aspects of potential toxicity in a limited toxicity evaluation in a single species, e.g., a repeated dose toxicity study of < 14 days duration that includes an evaluation of important functional endpoints (e.g., cardiovascular and respiratory).

In recent years, there has been much progress in the development of animal models that are thought to be similar to the human disease. These animal models include induced and spontaneous models of disease, gene knockout(s), and transgenic animals. These models may provide further insight, not only in determining the pharmacological action of the product, pharmacokinetics, and dosimetry, but may also be useful in the determination of safety (e.g., evaluation of undesirable promotion of disease progression). In certain cases, studies performed in animal models of disease may be used as an acceptable alternative to toxicity studies in normal animals. The scientific justification for the use of these animal models of disease to support safety should be provided.

1.2.2.3 Number / Gender of animals

The number of animals used per dose has a direct bearing on the ability to detect toxicity. A small sample size may lead to failure to observe toxic events due to observed frequency alone regardless of severity. The limitations that are imposed by sample size, as often is the case for

nonhuman primate studies, may be in part compensated by increasing the frequency and duration of monitoring. Both genders should generally be used or justification given for specific omissions.

1.2.2.4 Administration / Dose Selection

The route and frequency of administration should be as close as possible to that proposed for clinical use. Consideration should be given to pharmacokinetics and bioavailability of the product in the species being used and to the volume which can be safely and humanely administered to the test animals. Consideration should also be given to the effects of volume, concentration, formulation, and site of administration. The use of routes of administration other than those used clinically may be acceptable if the route must be modified due to limited bioavailability, limitations due to the route of administration, or to size/physiology of the animal species.

Dosage levels should be selected to provide information on a dose-response relationship, including a toxic dose and a no observed adverse effect level (NOAEL). For some classes of products with little to no toxicity, it may not be possible to define a specific maximum dose. In these cases, a scientific justification of the rationale for the dose selection and projected multiples of human exposure should be provided. To justify high dose selection, consideration should be given to the expected pharmacological/physiological effects, availability of suitable test material, and the intended clinical use. Where a product has a lower affinity to or potency in the cells of the selected species than in human cells, testing of higher doses may be important. The multiples of the human dose that are needed to determine adequate safety margins may vary with each class of biotechnology-derived pharmaceutical and its clinical indication(s).

1.2.2.5 Immunogenicity

Many biotechnology-derived pharmaceuticals intended for humans are immunogenic in animals. Therefore, measurement of antibodies associated with administration of these types of products should be performed when conducting repeated dose toxicity studies in order to aid in the interpretation of these studies. Antibody responses should be characterized (e.g., titer, number of responding animals, neutralizing or non-neutralizing) and their appearance should be correlated with any pharmacological and/or toxicological changes. Specifically, the effects of antibody formation on pharmacokinetic/pharmacodynamic parameters, incidence and/or severity of adverse effects, complement activation, or the emergence of new toxic effects should be considered when interpreting the data. Attention should also be paid to the evaluation of possible pathological changes related to immune complex formation and deposition.
In most cases, the immune response to biopharmaceuticals is variable, like that observed in humans. If the interpretation of the data from the safety study is not compromised by these issues, then no special significance should be ascribed to the antibody response.

The induction of antibody formation in animals is not predictive of a potential for antibody formation in humans. Humans may develop serum antibodies against humanized proteins, and frequently the therapeutic response persists in their presence. The occurrence of severe anaphylactic responses to recombinant proteins is rare in humans. In this regard, the results of guinea pig anaphylaxis tests, which are generally positive for protein products, are not predictive for reactions in humans; therefore, such studies are considered of little value for the routine evaluation of these types of products.

1.2.3 Specific Considerations

1.2.3.1 Safety Pharmacology

It is important to investigate the potential for undesirable pharmacological activity in appropriate animal models and, where necessary, to incorporate particular monitoring for these activities in the toxicity studies and/or clinical studies. Safety pharmacology studies measure functional indices of potential toxicity. These functional indices may be investigated in separate studies or incorporated in the design of toxicity studies. The aim of the safety pharmacology studies should be to reveal any functional effects on the major physiological systems (e.g., cardiovascular, respiratory, renal, and central nervous systems). Investigations may also include the use of isolated organs or other test systems not involving intact animals. All of these studies may allow for a mechanistically-based explanation of specific organ toxicities, which should be considered carefully with respect to human use and indication(s).

1.2.3.2 Exposure Assessment

1.2.3.2.1 Pharmacokinetics and Toxicokinetics

It is difficult to establish uniform guidance for pharmacokinetic studies for biotechnology-derived pharmaceuticals. Single and multiple dose pharmacokinetics, toxicokinetics, and tissue distribution studies in relevant species are useful; however, routine studies that attempt to assess mass balance are not useful. Differences in pharmacokinetics among animal species may have a significant impact on the productiveness of animal studies or on the assessment of dose-response relationships in toxicity studies.

Pharmacokinetic studies should, whenever possible, utilize preparations that are representative of those intended for toxicity testing and clinical use and employ a route of administration that is relevant to the anticipated clinical studies. Patterns of absorption may be influenced by formulation, concentration, site, and/or volume. Whenever possible, systemic exposure should be monitored during the toxicity studies.

When using radiolabeled proteins, it is important to show that the radiolabeled test material maintains activity and biological properties equivalent to that of the unlabeled material. Care should be taken in the interpretation of studies using radioactive tracers incorporated into specific amino acids because of recycling of amino acids into nondrug related proteins/peptides.

Some information on absorption, disposition, and clearance in relevant animal models should be available prior to clinical studies in order to predict margins of safety based upon exposure and dose.

1.2.3.2.2 Assays

The use of one or more assay methods should be addressed on a case-by-case basis and the scientific rationale should be provided. One validated method is usually considered sufficient. Ideally, the assay methods should be the same for animals and humans. The possible influence of plasma binding proteins and/or antibodies in plasma/serum on the assay performance should be determined.

1.2.3.2.3 Metabolism

The expected consequence of metabolism of biotechnology-derived pharmaceuticals is the degradation to small peptides and individual amino acids. Therefore, the metabolic pathways are generally understood. Classical biotransformation studies as performed for pharmaceuticals are not needed.

Understanding the behaviour of the biopharmaceutical in the biologic matrix (e.g., plasma, serum, cerebral spinal fluid) and the possible influence of binding proteins is important for understanding the pharmacodynamic effect.

1.2.3.3 Single Dose Toxicity Studies

Single dose studies may generate useful data to describe the relationship of dose to systemic and/or local toxicity. These data can be used to select doses for repeated dose toxicity studies. Information on dose-response relationships may be gathered through the conduct of a single dose toxicity study or as a component of pharmacology or animal model efficacy studies. The incorporation of safety pharmacology parameters in the design of these studies should be considered.

1.2.3.4 Repeated Dose Toxicity Studies

Repeated dose Toxicity studies should be designed considering the previous selection of animal species. The route and dosing regimen (e.g., daily versus intermittent dosing) should reflect the intended clinical use or exposure. When feasible, these studies should include toxicokinetics.

A recovery period should generally be included in study designs to determine the reversal or potential worsening of pharmacological/toxicological effects, and/or potential delayed toxic effects. For biopharmaceuticals that induce prolonged pharmacological/toxicological effects, recovery group animals should be monitored until reversibility is demonstrated. The duration of repeated dose studies should be based on the intended duration of clinical exposure and disease indication. This duration of animal dosing has generally been 1-3 months for most biotechnology derived pharmaceuticals.

For biopharmaceuticals intended for short-term use (e.g., < to 7 days) and for acute life-threatening diseases, repeated dose studies up to 2 weeks duration have been considered adequate to support clinical studies as well as marketing authorization. For those biopharmaceuticals intended for chronic indications, studies of 6 months duration have generally been appropriate, although in some cases shorter or longer durations have supported marketing authorizations. For biopharmaceuticals intended for chronic use, the duration of long-term toxicity studies should be scientifically justified.

1.2.3.5 Immunotoxicity Studies

Many biotechnology-derived pharmaceuticals are intended to stimulate or suppress the immune system and, therefore, may affect not only humoral but also cell-mediated immunity. Inflammatory reactions at the injection site may be indicative of a stimulatory response. It is important, however, to recognize that simple injection trauma and/or specific toxic effects caused by the formulation vehicle may also result in toxic changes at the injection site. In addition, the expression of surface antigens on target cells may be altered, which has

implications for autoimmune potential. Immunotoxicological testing strategies may require screening studies followed by mechanistic studies to clarify such issues. Routine tiered testing approaches or standard testing batteries, however, are not recommended for biotechnology-derived pharmaceuticals.

1.2.3.6 Reproductive Performance and Developmental Toxicity Studies

The need for reproductive/developmental toxicity studies is dependent upon the product, clinical indication and intended patient population. The specific study design and dosing schedule may be modified based on issues related to species specificity, immunogenicity, biological activity, and/or a long elimination half-life. For example, concerns regarding potential developmental immunotoxicity, which may apply particularly to certain monoclonal antibodies with prolonged immunological effects, could be addressed in a study design modified to assess immune function of the neonate.

1.2.3.7 Genotoxicity Studies

The range and type of genotoxicity studies routinely conducted for pharmaceuticals are not applicable to biotechnology-derived pharmaceuticals and therefore are not needed. Moreover, the administration of large quantities of peptides/proteins may yield uninterpretable results. It is not expected that these substances would interact directly with DNA or other chromosomal material. With some biopharmaceuticals, there is a potential concern about accumulation of spontaneously mutated cells (e.g., via facilitating a selective advantage of proliferation) leading to carcinogenicity. The standard battery of genotoxicity tests is not designed to detect these conditions. Alternative *in vitro* or *in vivo* models to address such concerns may have to be developed and evaluated.

1.2.3.8 Carcinogenicity Studies

Standard carcinogenicity bioassays are generally inappropriate for biotechnology-derived pharmaceuticals. However, product-specific assessment of carcinogenic potential may still be needed depending upon duration of clinical dosing, patient population, and/or biological activity of the product (e.g., growth factors, immunosuppressive agents, etc.). When there is a concern about carcinogenic potential, a variety of approaches may be considered to evaluate risk.

Incorporation of sensitive indices of cellular proliferation in long-term repeated dose toxicity studies may provide useful information. Careful consideration should be given to the selection of doses. The use of a combination of pharmacokinetic and pharmacodynamic endpoints with consideration of comparative receptor characteristics and intended human exposures represents the most scientifically based approach for defining the appropriate doses. The rationale for the selection of doses should be provided.

1.2.3.9 Local Tolerance Studies

Local tolerance should be evaluated. The formulation intended for marketing should be tested; however, in certain justified cases, the testing of representative formulations may be acceptable. In some cases, the potential adverse effects of the product can be evaluated in single or repeated

dose toxicity studies, thus obviating the need for separate local tolerance studies.

1.2.4 Addendum to S6

This addendum has been incorporated in the parent Guideline at the end of June 2011.

The purpose of the addendum is to complement, provide clarification on, and update the following topics discussed in ICH S6: species selection, study design, immunogenicity, reproductive and developmental toxicity, and assessment of carcinogenic potential. The recommendations of this addendum further harmonize the nonclinical safety studies to support the various stages of clinical development among the regions of European Union (EU), Japan, and the United States.
This guidance should facilitate the timely conduct of clinical trials, reduce the use of animals in accordance with the 3Rs (reduce/refine/replace) principles and reduce the use of other drug development resources. Although not discussed in this guidance, consideration should be given to the use of appropriate *in vitro* alternative methods for safety evaluation. These methods, if accepted by all ICH regulatory authorities, can be used to replace current standard methods.
This guidance promotes safe and ethical development and availability of new pharmaceuticals.
This addendum does not alter the scope of the original ICH S6 guidance. For biotechnology-derived products intended to be used in oncology the guidance *S9 Nonclinical Evaluation for Anticancer Pharmaceuticals* (ICH S9) should be consulted.

1.2.4.1 Species Selection

1.2.4.1.1 General Principles

A number of factors should be taken into account when determining species relevancy. Comparisons of target sequence homology between species can be an appropriate starting point, followed by *in vitro* assays to make qualitative and quantitative cross-species comparisons of relative target binding affinities and receptor/ligand occupancy and kinetics.
Assessments of functional activity are also recommended. Functional activity can be demonstrated in species-specific cell based systems and/or *in vivo* pharmacology or toxicology studies. Modulation of a known biologic response or of a pharmacodynamic (PD) marker can provide evidence for functional activity to support species relevance.
Consideration of species differences in target binding and functional activity in the context of the intended dosing regimen should provide confidence that a model is capable of demonstrating potentially adverse consequences of target modulation. When the target is expressed at very low levels in typical healthy preclinical species (e.g., inflammatory cytokines or tumor antigens), binding affinity and activity in cell-based systems can be sufficient to guide species selection.

In specific cases (i.e., where the approaches described above cannot be used to demonstrate a pharmacologically relevant species) tissue cross-reactivity (TCR) studies can be used to guide selection of toxicology species by comparison of tissue binding profiles between those human and animal tissues where target binding is expected.
As described in ICH S6, when no relevant species can be identified because the biopharmaceutical does not interact with the orthologous target in any species, use of homologous molecules or transgenic models can be considered.
For monoclonal antibodies and other related antibody products directed at foreign targets (i.e., bacterial, viral targets etc.), a short-term safety study (see *ICH S6*) in one species (choice of

species to be justified by the sponsor) can be considered; no additional toxicity studies, including reproductive toxicity studies, are appropriate. Alternatively, when animal models of disease are used to evaluate proof of principle, a safety assessment can be included to provide information on potential target-associated safety aspects. Where this is not feasible, appropriate risk mitigation strategies should be adopted for clinical trials.

1.2.4.1.2 One or two species

If there are two pharmacologically relevant species for the clinical candidate (one rodent and one non-rodent), then both species should be used for short-term (up to 1 month duration) general toxicology studies, then longer term general toxicity studies in one species are usually considered sufficient. The rodent species should be considered unless there is a scientific rationale for using non-rodents. Studies in two non-rodent species are not appropriate. The use of one species for all general toxicity studies is justified when the clinical candidate is pharmacologically active in only one species.

1.2.4.1.3 Use of Homologous Proteins

Use of homologous proteins is one of the alternative approaches described in ICH S6. Studies with homologous proteins can be used for hazard detection and understanding the potential for adverse effects due to exaggerated pharmacology, but are generally not useful for quantitative risk assessment; it can be possible to conduct safety evaluation studies using a control group and one treatment group for the study design and dose selected (e.g., maximum pharmacological dose).

1.2.4.2 Study Design

1.2.4.2.1 Dose Selection and application of PK/PD Principles

The toxicity of most biopharmaceuticals is related to their targeted mechanism of action; therefore, relatively high doses can elicit adverse effects, which are apparent as exaggerated pharmacology.

A rationale should be provided for dose selection taking into account the characteristics of the dose-response relationship. Pharmacokinetic-pharmacodynamic (PK-PD) approaches (e.g., simple exposure-response relationships or more complex modeling and simulation approaches) can assist in high dose selection by identifying:

1. a dose that provides the maximum intended pharmacological effect in the preclinical species;
2. a dose that provides an approximately 10-fold exposure multiple over the maximum exposure to be achieved in the clinic.

The higher of these two doses should be chosen for the high dose group in preclinical toxicity studies unless there is a justification for using a lower dose (e.g., maximum feasible dose).

Where *in vivo*/*ex vivo* PD endpoints are not available, the high dose selection can be based on PK data and available *in vitro* binding and/or pharmacology data. Corrections for differences in target binding and *in vitro* pharmacological activity between the nonclinical species and humans

should be taken into account to adjust the exposure margin over the highest anticipated clinical exposure.

1.2.4.2.2 Duration of Studies

For chronic use products, repeat dose toxicity studies of 6 months duration in rodents or non-rodents are considered sufficient; the principles for duration of toxicology studies are outlined in *ICH S9*.

1.2.4.2.3 Recovery

Recovery from pharmacological and toxicological effects with potential adverse clinical impact should be understood when they occur at clinically relevant exposures. This information can be obtained by an understanding that the particular effect observed is generally reversible/non-reversible or by including a non-dosing period in at least one study, to examine reversibility of these effects, not to assess delayed toxicity. The demonstration of complete recovery is not considered essential. The addition of a recovery period just to assess potential for immunogenicity is not required.

1.2.4.2.4 Exploratory Clinical Trials

The flexible approaches to support exploratory clinical trials as outlined in the guidance *M3(R2) Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals* (ICH M3(R2)) can be applicable to biopharmaceuticals. It is recommended that these approaches be discussed and agreed upon with the appropriate regulatory authority.

1.2.4.3 Immunogenicity

Such analyses in nonclinical animal studies are not relevant in terms of predicting potential immunogenicity of human or humanized proteins in humans.

Measurement of anti-drug antibodies (ADA) in nonclinical studies should be evaluated when there is:

1. evidence of altered PD activity;
2. unexpected changes in exposure in the absence of a PD marker;
3. evidence of immune-mediated reactions (immune complex disease, vasculitis, anaphylaxis, etc.).

Characterization of neutralizing potential is warranted when ADAs are detected and there is no PD marker to demonstrate sustained activity in the *in vivo* toxicology studies. Neutralizing antibody activity can be assessed indirectly with *ex-vivo* bioactivity assay or an appropriate combination of assay formats for PK-PD, or directly in a specific neutralizing antibody assay.

1.2.4.4 Reproductive and Developmental Toxicity

Reproductive toxicity studies should be conducted in accordance with the principles outlined in the guidance *S5(R2) Detection of Toxicity to Reproduction for Medicinal Products and Toxicity*

to Male Fertility (ICH S5).

The evaluation of toxicity to reproduction should be conducted only in pharmacologically relevant species. When the clinical candidate is pharmacologically active in rodents and rabbits, both species should be used for embryo-fetal development (EFD) studies, unless embryo-fetal lethality or teratogenicity has been identified in one species.

Developmental toxicity studies should only be conducted in nonhuman primates (NHPs) when they are the only relevant species. When the clinical candidate is pharmacologically active only in NHPs, there is still a preference to test the clinical candidate. However, an alternative model can be used in place of NHPs if appropriate scientific justification is provided.
When no relevant animal species exists for testing the clinical candidate, the use of transgenic mice can be considered. For products that are directed at a foreign target such as bacteria and viruses, in general, no reproductive toxicity studies would be expected.

1.2.4.4.1 Fertility

For products where mice and rats are pharmacologically relevant species, an assessment of fertility can be made in one of these rodent species (see ICH S5).

It is recognized that mating studies are not practical for NHPs. However, when the NHP is the only relevant species, the potential for effects on male and female fertility can be assessed by evaluation of the reproductive tract (organ weights and histopathological evaluation) in repeat dose toxicity studies of at least 3 months' duration using sexually mature NHPs. If there is a specific cause for concern based on pharmacological activity or previous findings, specialized assessments such as menstrual cyclicity, sperm count, sperm morphology/motility, and male or female reproductive hormone levels can be evaluated in a repeat dose toxicity study.

If there is a specific concern from the pharmacological activity about potential effects on conception/implantation and the NHP is the only relevant species, the concern should be addressed experimentally. A homologous product or transgenic model could be the only practical means to assess potential effects on conception or implantation when those are of specific concern. However, it is not recommended to produce a homologous product or transgenic model solely to conduct mating studies in rodents.

1.2.4.4.2 Embryo-Fetal Development (EFD) and Pre/Post-Natal Development (PPND)

Potential differences in placental transfer of biopharmaceuticals should be considered in the design and interpretation of developmental toxicity studies. For products pharmacologically active only in NHPs, several study designs can be considered based on intended clinical use and expected pharmacology. One well-designed study in NHPs that includes dosing from day 20 of gestation to birth (enhanced PPND; ePPND) can be considered, rather than separate EFD and/or PPND studies.

For the single ePPND study design described above, no Caesarian section group is warranted, but assessment of pregnancy outcome at natural delivery should be performed. This study should also evaluate offspring viability, external malformations, skeletal effects (e.g., by X-ray) and, ultimately, visceral morphology at necropsy. Ultrasound is useful to track maintenance of pregnancy but is not appropriate for detecting malformations. These latter data are derived from postpartum observations. Because of confounding effects on maternal care of offspring, dosing

of the mother postpartum is generally not recommended. Other endpoints in the offspring can also be evaluated if relevant for the pharmacological activity. The duration of the postnatal phase will be dependent on which additional endpoints are considered relevant based on mechanism of action.

The developmental toxicity studies in NHP as outlined above are just hazard identification studies; therefore, it might be possible to conduct these studies using a control group and one dose group, provided there is a scientific justification for the dose level selected. An example of an appropriate scientific justification would be a monoclonal antibody that binds a soluble target with a clinical dosing regimen intended to saturate target binding.

1.2.4.4.3 Timing of Studies

If women of child-bearing potential are included in clinical trials prior to acquiring information on effects on embryo-fetal development, appropriate clinical risk management is appropriate, such as use of highly effective methods of contraception (see ICH M3(R2)).

For biopharmaceuticals pharmacologically active only in NHPs, where there are sufficient precautions to prevent pregnancy (see ICH M3(R2), section XI.C (11.3), paragraph 2), an EFD or ePPND study can be conducted during Phase III, and the report submitted at the time of marketing application.

If the rodent or rabbit is a relevant species, see ICH M3(R2) for timing of reproductive toxicity studies. ICH M3(R2) should also be followed for the timing of data on fertility for products where rodents are relevant species.

For oncology products which fall within the scope of ICH S9, see that guidance for aspects relating to timing of study conduct.

1.2.4.5 Carcinogenicity

The need for a product-specific assessment of the carcinogenic potential for biopharmaceutical should be determined with regard to the intended clinical population and treatment duration (see the guidance *S1A The Need for Carcinogenicity Studies of Pharmaceuticals*).

This strategy could be based on a weight of evidence approach, including a review of relevant data from a variety of sources. The data sources can include published data (e.g., information from transgenic, knock-out or animal disease models, human genetic diseases), information on class effects, detailed information on target biology and mechanism of action, *in vitro* data, data from chronic toxicity studies and clinical data. In some cases, the available information can be sufficient to address carcinogenic potential and inform clinical risk without additional nonclinical studies.

For products where there is insufficient knowledge about specific product characteristics and mode of action in relation to carcinogenic potential, a more extensive assessment might be appropriate (e.g., understanding of target biology related to potential carcinogenic concern, inclusion of additional endpoints in toxicity studies). The product-specific assessment of carcinogenic potential is used to communicate risk and provide input to the risk management plan along with labeling proposals, clinical monitoring, post-marketing surveillance, or a combination of these approaches.

Rodent bioassays (or short-term carcinogenicity studies) with homologous products are generally of limited value to assess carcinogenic potential of the clinical candidate. Alternative approaches can be considered as new strategies/assays are developed.
See references [52] [53] [54] [55] [56] [57] and [58].

2. SELECTION OF THE RELEVANT ANIMAL SPECIES

The cytokine IL-2 is synthetized and produced after antigenic stimulation of resting T cells.
T cells use IL-2 for clonal expansion and cytokine production. IL-2 binds to its receptor, the IL-2 receptor (IL-2R).

The high affinity IL-2 is a heterotrimeric cell surface receptor composed of α, β and γ-polypeptide chains. The IL-2Rα chain (also known as TAC, CD25) is specific for the IL-2R, but β and γ chains are part of cytokine receptor superfamily and are also shared by other cytokine receptors [59]. The intermediate affinity receptor is a dimer composed of β and γ chains while the low affinity receptor consist of a monomeric α. Subunit that has no signal transduction capacity [67]. Resting T-cells, B-cells, and monocytes express few IL-2Rα-subunit but the receptor is rapidly transcribed and expressed upon activation [61]. Cells expressing the high affinity IL-2R express the IL-2Rα-subunits in excess, which leads to both high and low affinity IL-2 binding profiles [62] [63].

IL-2Rα is highly expressed by T cells in some autoimmune diseases (e.g. rheumatoid arthritis, scleroderma, uveitis), skin disorders (psoriasis, atopic dermatitis), and a variety of lymphoid neoplasms (e.g. T-cell leukemia, Hodgkin's disease) [64].
Moreover, IL-2Rα expression is associated with allograft rejection and graft-versus-host responses (GvHD) [65], [66] **Error! Reference source not found.**.

Fully human monoclonal antibody against IL-2Rα was generated aiming of preventing the biological consequences of IL-2 binding to its receptor.

Selection of the relevant animal species for preclinical safety studies was based on:

- interspecies amino-acid sequence homology of CD25 extracellular domain as obtained from the NCBI Basic Local Alignment Search Tool (BLAST);
- cross-reactivity of *Anti-CD25 mAb*, fully human anti-CD25 antibody (lead candidate), evaluated in Peripheral Blood Mononuclear Cells (PBMCs) from different animal species.

2.1 Selection of the relevant animal species by amino-acid sequence homology

The BLAST program revealed 91% sequence identity between human and macaque monkeys. In contrast, percentages of sequence identity drop down when comparing the human sequence with other species: dog (61%), pig (56%), mouse (60%) and rat (60%).

Cross reactivity was therefore more likely to occur between human and the macaque monkey rather than with the other above-mentioned species.

Moreover, published data [67] on comparative sequence analyses suggest that both human CD25 and Rhesus CD25 share identity for most of the critical amino acids essential for viable folding and IL-2 ligand binding.

2.2 Characterization of relevant animal species by cross-reactivity

Extensive laboratory work was conducted to comparatively evaluate the capacity of *Anti- CD25 mAb* to bind monkey (Rhesus, Cynomolgus and marmoset), minipig, mouse, rat and rabbit CD25 receptor expressed on CD3+ T cells using resting or activated Peripheral Blood Mononuclear Cells (PBMCs).

PBMCs obtained from blood samples (purchased externally) from different animal species.
The following animal species were used and suppliers are shown in brackets:

- Macaca fascicularis (Cynomolgus monkey) (Hartelust, The Netherland);
- Macaca mulatta (Rhesus monkey) (Hartelust, The Netherland);
- Marmoset (Callithrix jacchus) (Covance Laboratories GmbH);
- Minipigs (Ellegaard Göttingen);
- New Zealand white rabbits (Charles River Italy);
- Sprague Dawley Crl:CD(SD) BR rats (Charles River Italy);
- Crl:CD-1 (ICR) BR mice (Charles River Italy).

PBMCs are cells in which IL-2Rα is expressed after antigenic stimulation and this expression can be easily monitored.

Resting and activated animal PBMCs were used for testing their expression of CD25 antigen.
In addition, resting and activated human PBMCs were assayed in the same way as control of assay performance and to comparatively evaluate the binding capacity of test item to non-human blood cells.

2.3 Commercial monoclonal anti-CD25 antibodies

Daclizumab (Zenapax, Roche). A humanized IgG1 IL-2Rı. antagonist.
Basiliximab (Simulect, Novartis). A chimeric human and mouse IgG1 IL-2Rı. antagonist.

The above commercial therapeutic monoclonal antibodies were used in parallel with the test item to assess the cross-reactivity equivalence of *Anti-CD25 mAb.*
The commercial monoclonal antibodies were tested at the same concentrations and in the same vehicle as the test item.

2.4 Cross – reactivity evaluation: methods

The binding ability of *Anti-CD25 mAb* to IL-2Rα, expressed on PBMCs obtained from whole blood samples of different animal species, were evaluated by indirect immunofluorescence method in flow cytometry.
Blood samples from one human healthy donor, and two healthy animals from each species were tested.

2.4.1 PBMCs isolation

Fresh heparinized peripheral blood was diluted with PBS at room temperature. The cell suspensions, well mixed, were added directly to Ficoll gradient and centrifuged at 1000 g for 20 min at 20 °C. After centrifugation the mononuclear cells were harvested at the interface ring, washed once and suspended in complete medium at a concentration of $2x10^6$ cells/mL.

2.4.2 PBMCs stimulation

Cell suspensions at the concentration of $2x10^6$ cells/mL were transferred into culture flasks or 24-well plates, depending on the amount of PBMCs recovered, to obtain the final concentration of $1x10^6$ cells/mL. Cells were exposed to phytohemagglutinin (PHA : 5 µg/mL) for 3 days at 37 °C in a humidified 5% CO_2 incubator.

2.4.3 Resting and activated PBMC staining for flow cytometry

Resting PBMCs from all animal species tested were stained for flow cytometry on the day of PBMCs isolation. Cells were suspended at a concentration of $2x10^6$ cells/mL. CD25 expression was evaluated using *Anti-CD25 mAb* (the test item), commercial monoclonal anti-CD25 antibodies (Zenapax and Simulect) or isotype IgG control at the final concentrations of 100 and 10 µg/mL.
Initially, the concentrations of 100 and 10 µg/mL were also used to investigate the crossreactivity of the *Anti-CD25 mAb*, commercial mAbs (Zenapax and Simulect) or isotype IgG control with stimulated PBMCs from marmosets, minipigs, rabbits, rats and mice.

Activated PBMCs were harvested at the end of the stimulation period, centrifuged once (7 min at 300 g), counted and suspended at the concentration of $2x10^6$ cells/mL. $1x10^5$ cells/50 µL of PBS + 0.1% BSA were dispensed into plastic conical tubes and incubated with 50 µL of the primary antibodies (30 min, 4°C) at different concentrations.
After washing twice in PBS, the cells were incubated for 30 min at 4°C with 50 µL of a second antibody mixture containing rabbit anti-human IgG-FITC or PE conjugated and anti species-specific CD3 appropriately conjugated.
The cells were washed once, suspended in 500 µL of PBS and cell-associated fluorescence was assessed by flow cytometry using FACSCanto and FACSDiva software (BD Biosciences) with a gate on CD3+ T-cells for all species with the exception of rabbits in which the gate was done on CD4+ T-cells.

The binding capacity of the antibodies to IL-2Rα was expressed as MFI of CD3+ or CD4+ cells. MFI data obtained from Cynomolgus and Rhesus monkeys, and human donor were used to calculate EC_{50} (half maximal effective concentration) values with Graph Pad prism software (GraphPad Software, Inc. La Jolla, CA, USA).

2.5 Cross- reactivity evaluation: results

The ability of *Anti-CD25 mAb* to bind the CD25 receptor was assessed on resting and activated PBMCs and compared with two marketed therapeutic anti-CD25 antibodies, Zenapax

(Daclizumab) and Simulect (Basiliximab).
The same experiment in each species was performed, for control and comparison, on resting and activated human PBMCs.
In each assay the expression of CD25 antigen on resting and activated PBMCs was also evaluated with commercial mAbs for research use only, specific for each animal species used.

Cynomolgus monkey was the only species where binding to CD25-bearing cells was shown at potentially relevant *in vivo* concentrations. Therefore the Cynomolgus monkey was considered a valuable candidate for the preclinical development program of *Anti-CD25 mAb* .

Data subsequently obtained in the *in vitro*/*ex-vivo* functional assays (e.g. inhibition of anti-CD3-stimulated PBMCs proliferation), coupled with the CD25 saturation data of the CD4+ T cells from the *in vivo* experiments collectively allowed to qualify the Cynomolgus monkey as a responsive animal species to *Anti- CD25 mAb*.

The overall results indicated that the CD25 expression, evaluated as CD25+ cell percentage or MFI, was absent or very low on human or animal resting PBMCs, while it was expressed on activated PBMCs, indicating that the method used was suitable for the investigation purpose.

2.5.1 Cynomolgus monkey

Anti- CD25 mAb cross-reactivity was assessed on Cynomolgus monkey resting and activated PBMCs.

Anti- CD25 mAb, commercial mAbs (Zenapax and Simulect) or isotype IgG control were used at the concentrations of 100 and 10 µg/mL on resting PBMCs, while different concentrations were tested on activated PBMCs.

The assays were performed on two animals (1 male and 1 female) and on one human being.

No binding was observed on monkey and human resting PBMCs (***Figures 4, 5 and 6***).

Regarding activated monkey PBMCs (***Figures 7, 8 and 9***), *Anti-CD25 mAb* binding capacity was relevant compared to that shown by the negative control (IgG1k), but was lower than the binding capacity shown by Zenapax and Simulect.

Zenapax and Simulect binding was obtained at mAb concentrations from 1.5 to 100 ng/mL, while *Anti-CD25 mAb* bound primate PBMCs at antibody concentrations from 390 ng/mL to 100000 ng/mL.

On human cells, *Anti-CD25 mAb* bound IL-2Rα in a dose-dependent manner more efficiently than on monkey cells, and the binding capacity was almost comparable with Zenapax and Simulect tested in the same conditions.

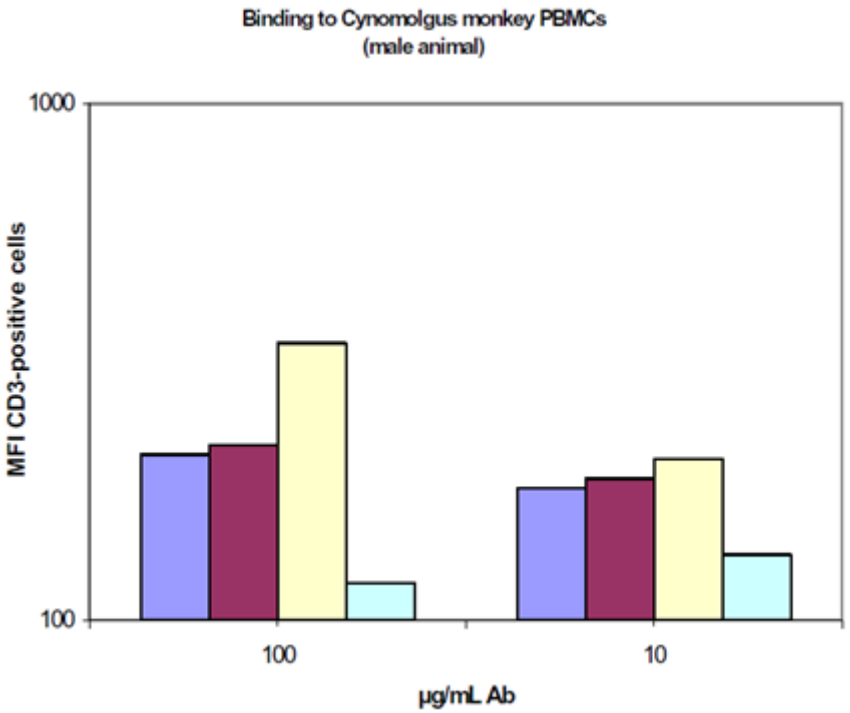


Fig. 4: *Cross reactivity evaluation in resting Cynomolgus Monkey PBMCs (male)*
Cross reactivity evaluation of Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) and Zenapax competitor 2(purple) to resting Cynomolgus monkey PBMCs (isotype control: light blue).

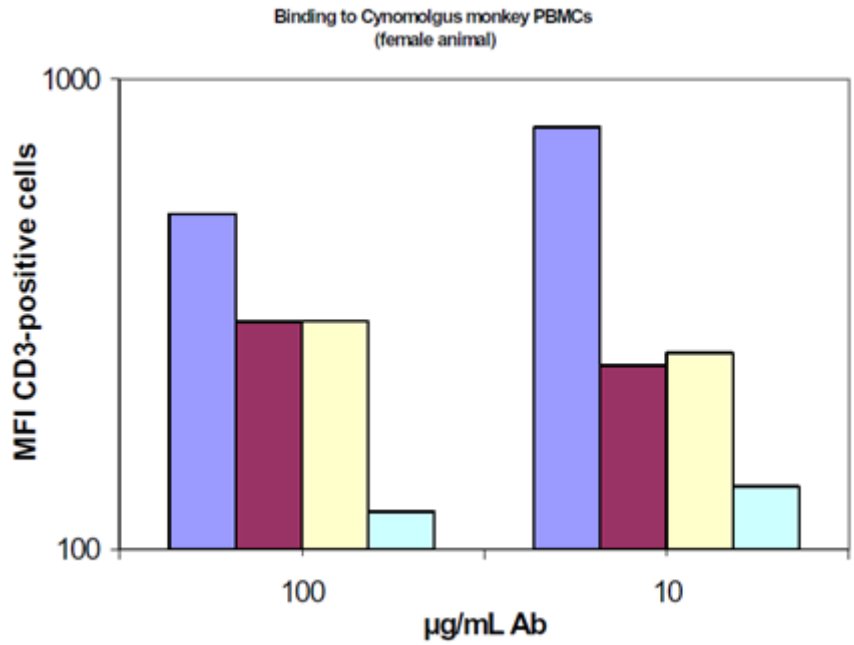


Fig. 5: *Cross reactivity evaluation in resting Cynomolgus Monkey PBMCs (female)*
Cross reactivity evaluation of Anti-CD25(violet), Simulect competitor 1(Yellow) and Zenapax competitor 2(purple) to resting Cynomolgus monkey PBMCs (isotype control: light blue).

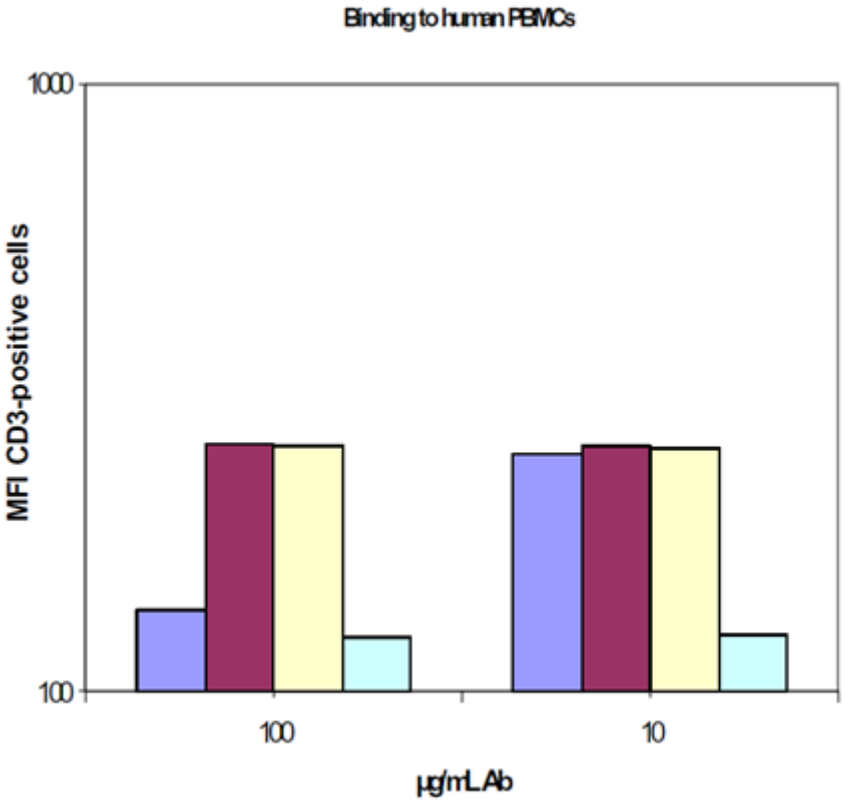


Fig. 6: *Cross reactivity evaluation in <u>resting</u> human PBMCs*
Cross reactivity evaluation of Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) and Zenapax competitor 2(purple) to resting human PBMCs (isotype control: light blue).

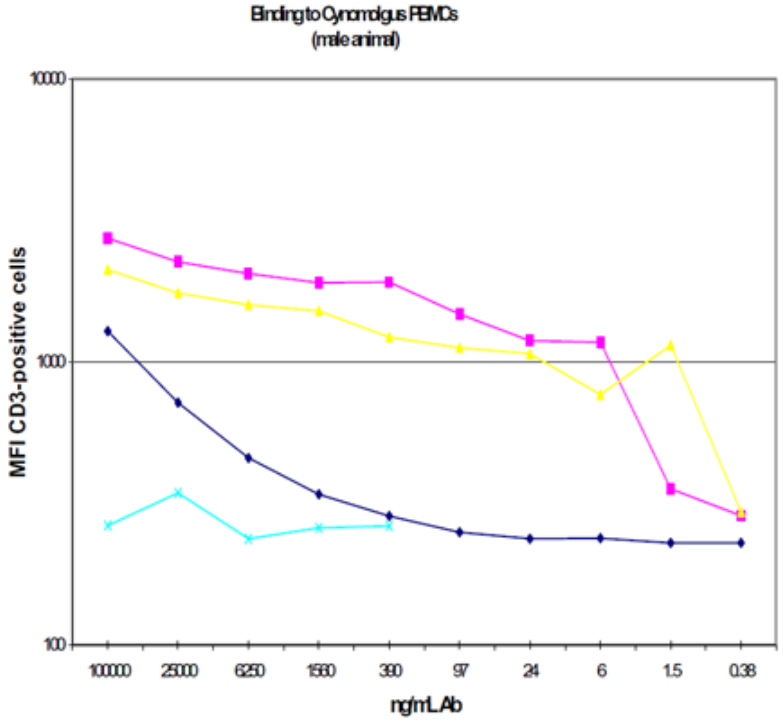


Fig. 7: *Cross reactivity evaluation in <u>activated</u> Cynomolgus Monkey PBMCs (male)*
Cross-reactivity evaluation of Anti-CD25 mAb (blue), Simulect competitor 1(Yellow) and Zenapax competitor 2(pink) to activated Cynomolgus monkey PBMCs (FITC MFI of Abs binding on CD3+ cells), (isotype control: light

blue).

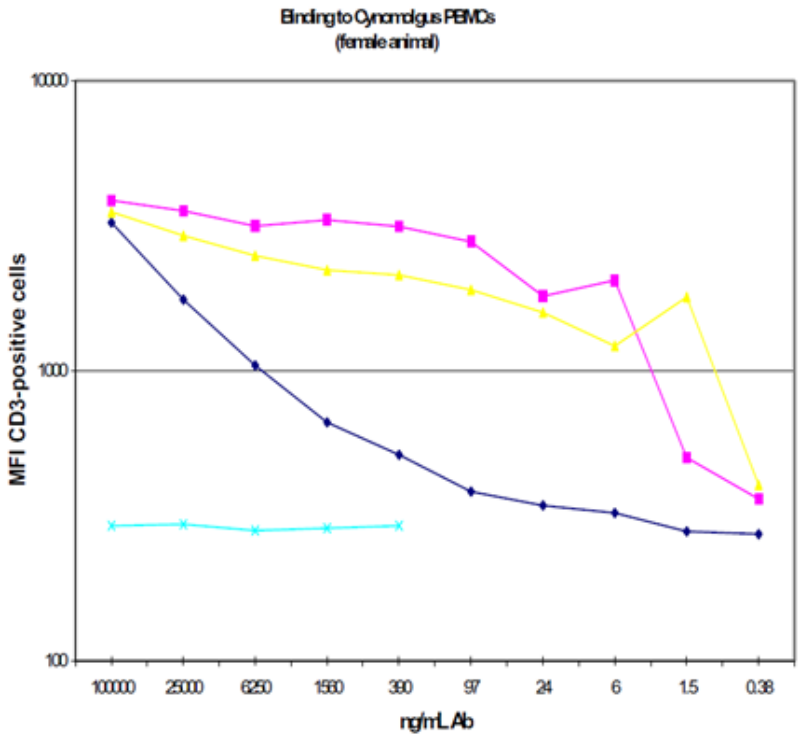


Fig. 8: *Cross reactivity evaluation in activated Cynomolgus Monkey PBMCs (female)*
Cross-reactivity evaluation of Anti-CD25 mAb (blue), Simulect competitor 1(Yellow) and Zenapax competitor 2(pink) to activated Cynomolgus monkey PBMCs (FITC MFI of Abs binding on CD3+ cells), (isotype control: light blue).

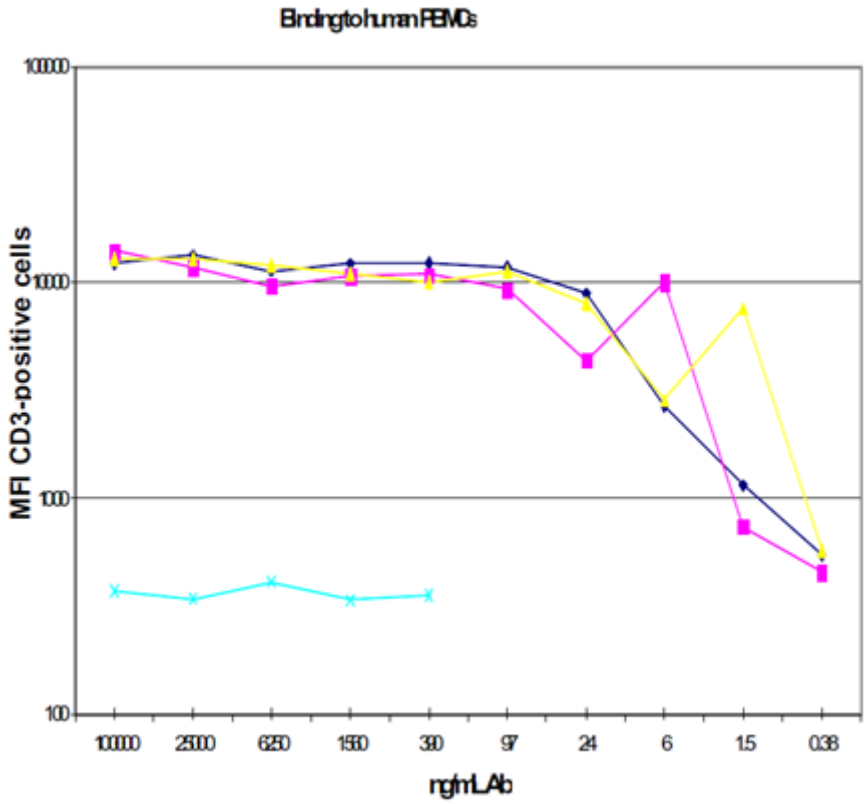


Fig. 9: *Cross reactivity evaluation in activated human PBMCs*
Cross-reactivity evaluation of Anti-CD25(blue), Simulect competitor 1(Yellow) and Zenapax competitor 2(pink) to activated human PBMCs (FITC MFI of Abs binding on CD3+ cells), (isotype control: light blue).

Anti- CD25 mAb, Simulect (competitor 1) and Zenapax (competitor 2) highest concentrations were tested on activated monkey PBMCs in order to obtain a saturation curve and to calculate the EC_{50} values (***Figures 10 and 11***).

PBMCs from 2 male monkeys were used, and the following concentrations were tested: 500000, 250000, 125000, 20800, 3500, 580, 96, 16, 2.7, 0.45 ng/mL. As shown in *Figures 6a* and *6b*, the full saturation curve was reached both for Anti-CD25 and the two competitors mAbs at the concentration of 125 and 250 µg/mL. The reduced binding at the concentration of 500 µg/mL was due to an antibody excess zone. On the basis of the results obtained, data from the cross-reactivity evaluation in activated PBMCs, were used to calculate EC_{50}s of the three products since 100 µg/mL was also considered as a saturating concentration.

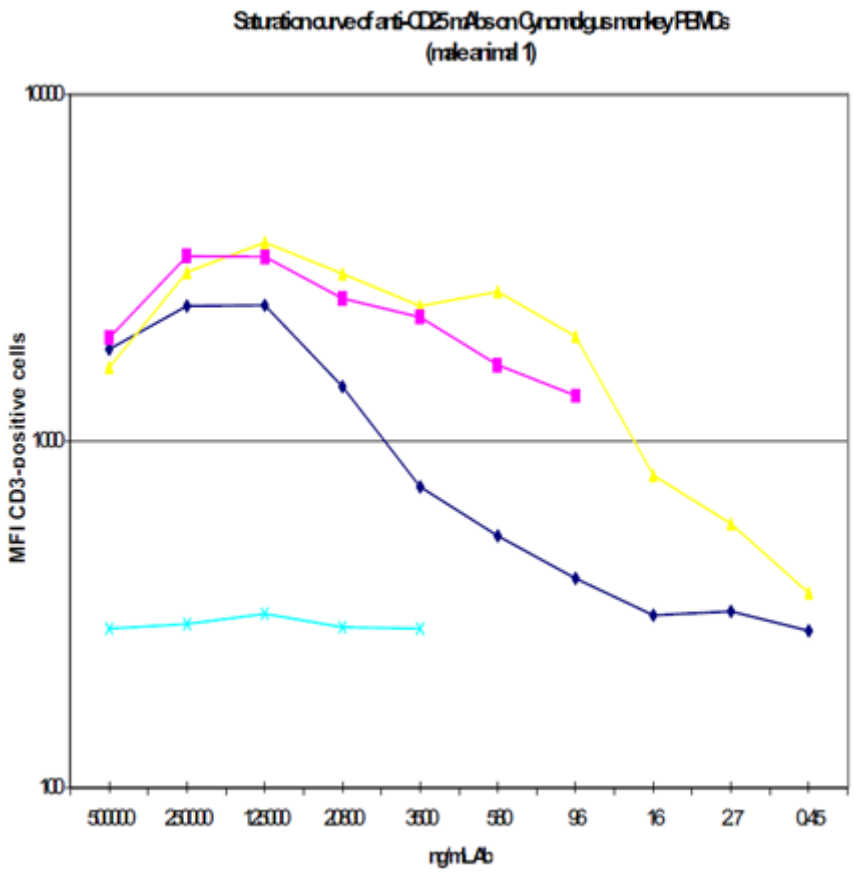


Fig. 10: *Saturation curve in Cynomolgus monkey activated PBMCs (male 1)*
Saturation curve of the binding of Anti- CD25 mAb (blue), competitor 1(yellow) and competitor 2(pink) to Cynomolgus monkey activated PBMCs. (FITC MFI of Abs binding on CD3+ cells)

Note: *Competitor 2(pink)* data from the 3 lowest concentrations are not shown (unreliable due to a technical error in sample dilution.)

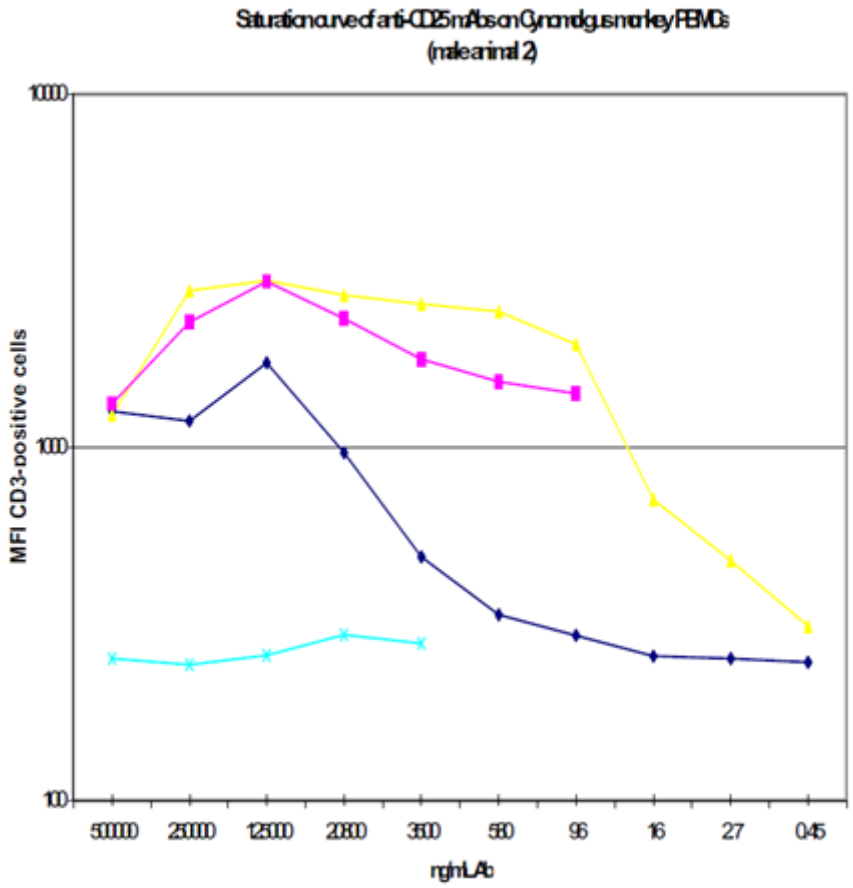


Fig. 11: *Saturation curve in Cynomolgus monkey activated PBMCs (male 2)*
Saturation curve of the binding of Anti- CD25 mAb (blue), competitor 1(yellow) and competitor 2(pink) to Cynomolgus monkey activated PBMCs. (FITC MFI of Abs binding on CD3+ cells)

Note: *Competitor 2(pink)* data from the 3 lowest concentrations are not shown (unreliable due to a technical error in sample dilution.)

The results obtained from *Anti-CD25 mAb* cross-reactivity evaluation in PBMCs showed that cross-reactivity with Cynomolgus monkey PBMCs was lower and the EC_{50} was higher than those determined for the two competitors.

2.5.2 Rhesus monkeys

Anti-CD25 mAb cross-reactivity was assessed on Rhesus monkey resting and activated PBMCs with the test procedure and reagents used for Cynomolgus monkeys.

Anti-CD25 mAb, commercial mAbs (Zenapax and Simulect) or isotype IgG control were used at the concentration range of 100000 to 0.38 ng/mL on activated monkey (1 male and 1 female) and human PBMCs.

No binding was observed on monkey and human resting PBMCs (***Figures 12, 13 and 14***).

Regarding activated monkey PBMCs (***Figures 15, 16 and 17***) *Anti-CD25 mAb* binding capacity was relevant compared to that shown by the negative control (IgG1k), but was lower than the binding capacity shown by Zenapax and Simulect. Zenapax and Simulect binding was obtained at mAb concentrations from 1.5 to 100 ng/mL, while *Anti-CD25 mAb* bound primate PBMCs at

antibody concentration from 390 ng/mL to 100000 ng/mL.

The results obtained in this assay were used to calculate EC_{50s} of the three products since 100 µg/mL was considered as a saturating concentration due to the binding activity very similar to that observed in Cynomolgus PBMCs.

On human cells, *Anti-CD25 mAb* bound IL-2Rα in a dose-dependent manner more efficiently than on monkey cells, and the binding capacity was almost comparable with Zenapax and Simulect tested in the same conditions, confirming the results obtained in the Cynomolgus assay.

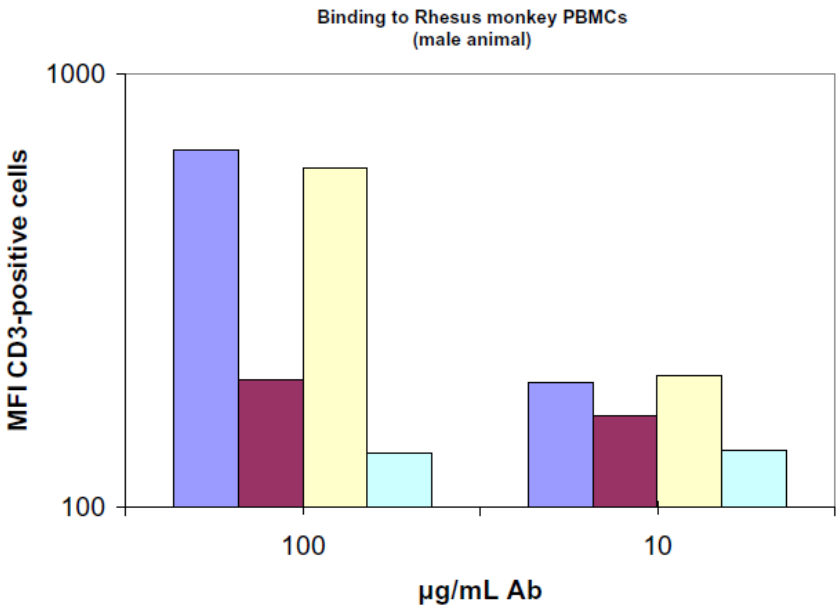


Fig. 12: *Cross reactivity evaluation in resting Rhesus Monkey PBMCs (male)*
Cross reactivity evaluation of Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) and Zenapax competitor 2(purple) to Rhesus monkey resting PBMCs (isotype control: light blue).

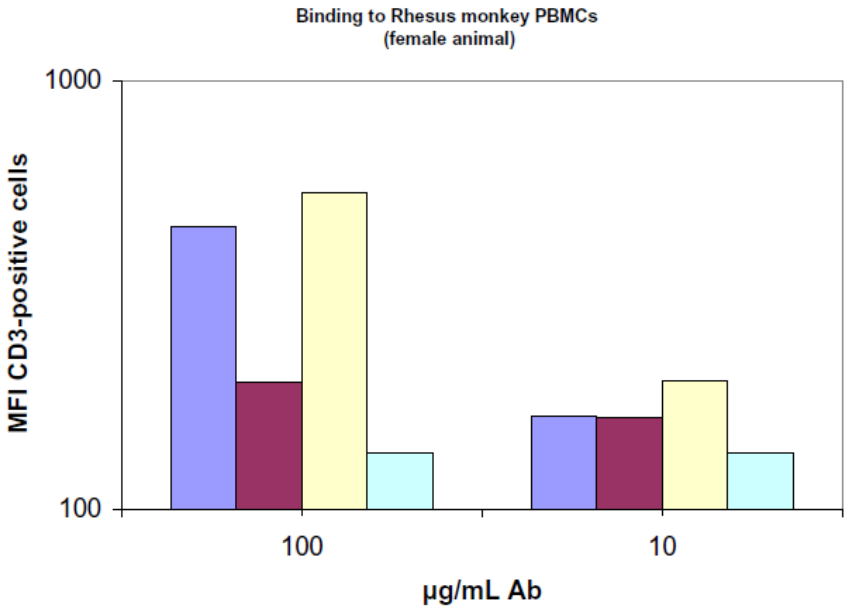


Fig. 13: *Cross reactivity evaluation in resting Rhesus Monkey PBMCs (female)*

Cross reactivity evaluation of Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) and Zenapax competitor 2(purple) to Rhesus monkey resting PBMCs (isotype control: light blue).

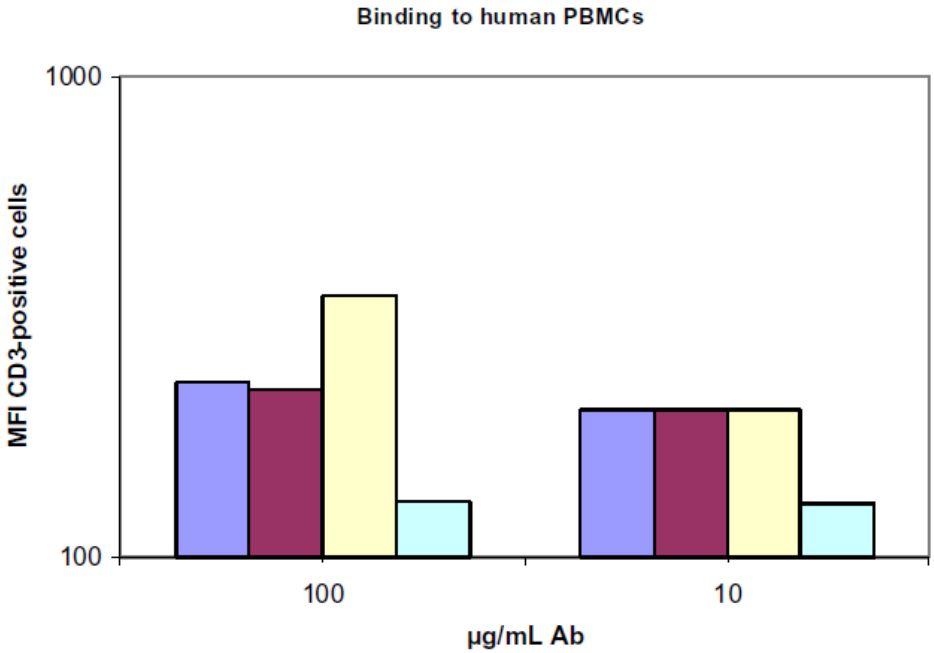


Fig. 14: *Cross reactivity evaluation in resting human PBMCs*
Cross reactivity evaluation of Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) and Zenapax competitor 2(purple) to human resting PBMCs (isotype control: light blue).

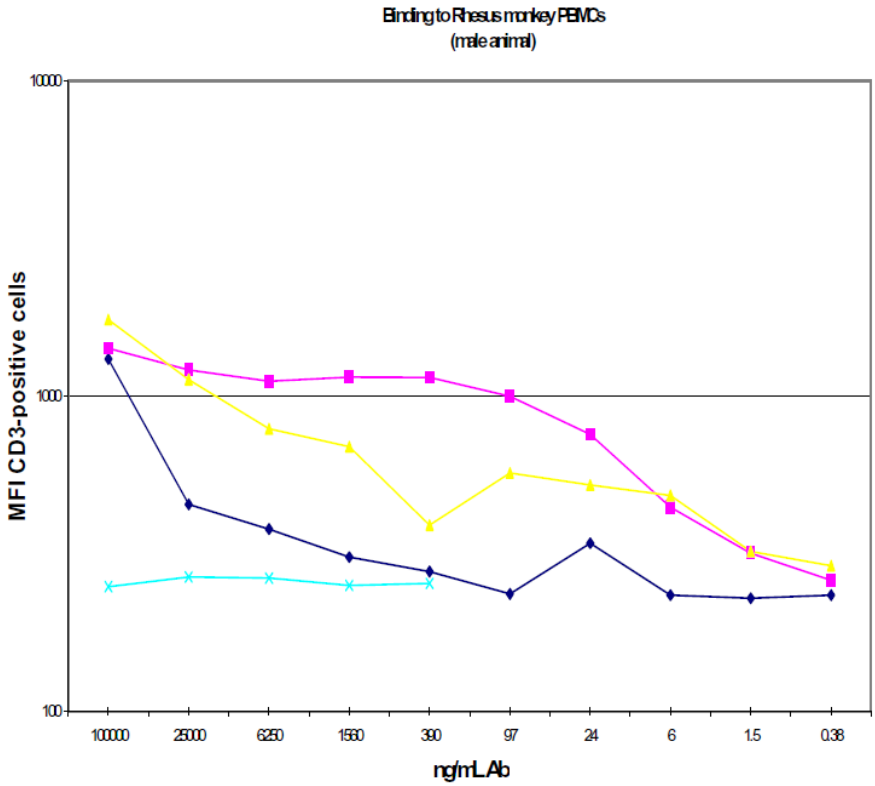


Fig. 15: *Cross reactivity evaluation in activated Rhesus Monkey PBMCs (male)*
Cross-reactivity evaluation of Anti-CD25(blue), Simulect competitor 1(Yellow) and Zenapax competitor 2(pink) to activated Rhesus monkey PBMCs (FITC MFI of Abs binding on CD3+ cells), (isotype control: light blue).

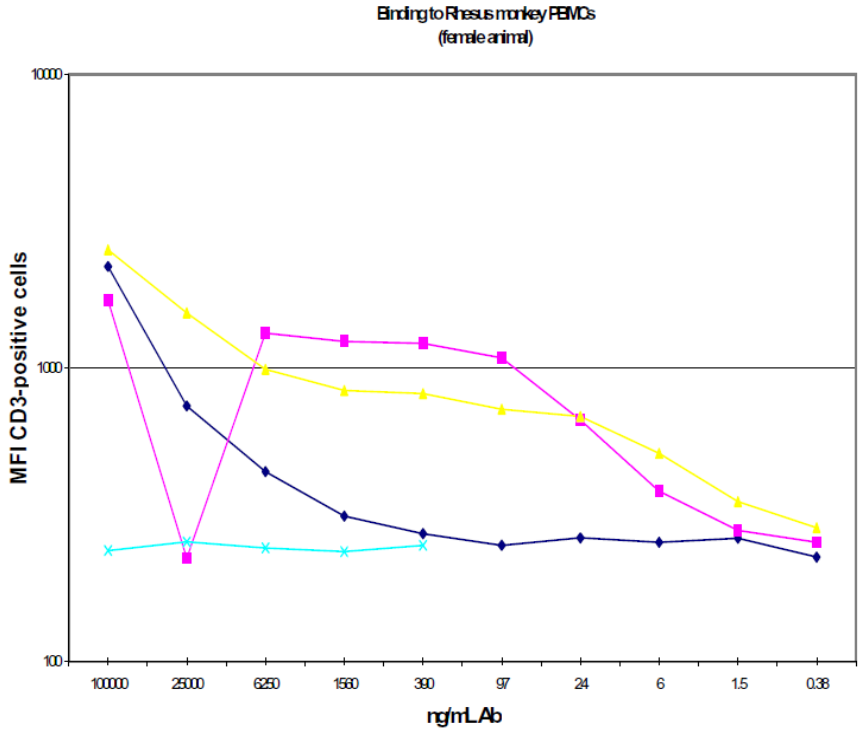


Fig. 16: *Cross reactivity evaluation in activated Rhesus Monkey PBMCs (female)*
Cross-reactivity evaluation of Anti-CD25(blue), Simulect competitor 1(Yellow) and Zenapax competitor 2(pink) to activated Rhesus monkey PBMCs (FITC MFI of Abs binding on CD3+ cells), (isotype control: light blue).

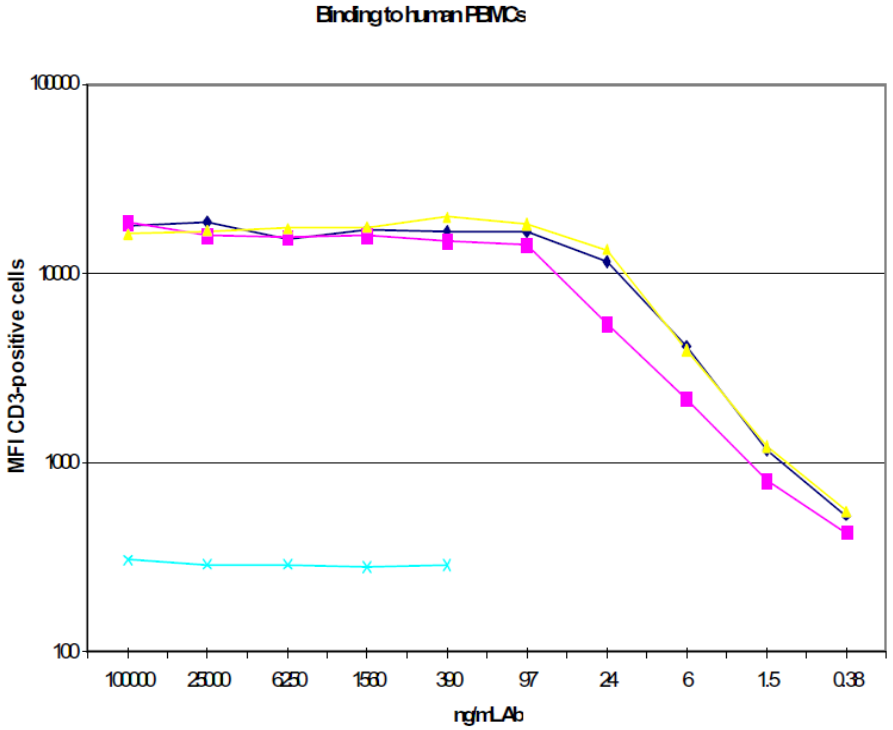


Fig. 17: *Cross reactivity evaluation in activated human PBMCs*
Cross-reactivity evaluation of Anti-CD25(blue), Simulect competitor 1(Yellow) and Zenapax competitor 2(pink) to activated humanPBMCs (FITC MFI of Abs binding on CD3+ cells), (isotype control: light blue).

Note: The lack of Zenapax binding in the female animal at the concentration of 25000 ng/mL was due to a technical error.

2.5.3 *Anti-CD25 mAb*, Zenapax and Simulect EC50 estimation on Cynomolgus and Rhesus monkey and human activated PBMCs

MFI data obtained from Cynomolgus and Rhesus monkey, and human activated PBMCs in the various assays were used to calculate EC_{50} values. Individual and mean values are reported in ***Table 1*** and ***2***, respectively.

Anti-CD25 mAb bound to the Cynomolgus activated PBMCs with an EC_{50} value of about 18000 ng/mL. *Anti-CD25 mAb* EC_{50} to Cynomolgus CD25 was about 90 times higher than Simulect (207 ng/mL) and 165 times higher than Zenapax (110 ng/mL), indicating a lower binding capacity than the commercial anti-CD25 mAbs. In comparison to human PBMCs, the *Anti-CD25 mAb* EC_{50} to the Cynomolgus CD25 was 1000 times higher.
Regarding Rhesus animals, *Anti-CD25 mAb* bound to the Rhesus PBMCs with an EC_{50} value of about 33000 ng/mL, indicating that the *Anti-CD25 mAb* binding ability to Rhesus CD25 receptor was about 2 times less than Cynomolgus in comparison to human PBMC. Zenapax EC_{50} (118 ng/mL) was similar to that obtained in Cynomolgus, while the Simulect EC_{50} of 9634 ng/mL was about 50 times higher.
Anti-CD25 mAb and Simulect bound to the human PBMCs with a mean EC_{50} of 17 and 18 ng/mL respectively. Zenapax binding ability was 4 fold less than *Anti-CD25 mAb* with an EC_{50} of 73 ng/mL.
From the calculation of the EC_{50} values of the anti-CD25 antibodies against human CD25 it can be concluded that the binding capacity of *Anti-CD25 mAb* and Simulect are similar, and that *Anti-CD25 mAb* binds 4 times as much as Zenapax.

Table 1: EC_{50} individual data (ng/mL)

Binding of Anti-CD25 mAb, Simulect and Zenapax to activated PBMCs			
	EC_{50} individual values (ng/mL)		
	Anti-CD25 mAb	**Simulect**	**Zenapax**
Male Cynomolgus	21410	193.1	174.4
Female Cynomolgus	17641	224.9	46.37
Male 1 Cynomolgus	14460	325.0	-
Male 2 Cynomolgus	18697	84.06	-
Male Rhesus	34103	10875	74.15
Female Rhesus	32800	8393	161.3
Human donor	16.93	20.93	91.77
Human donor	17.64	15.72	54.74

Table 2: Mean EC_{50} (ng/mL)

Binding of Anti-CD25 mAb, Simulect and Zenapax to activated PBMCs			
	Mean EC_{50} (ng/mL)		
	Anti-CD25 mAb	**Simulect**	**Zenapax**
Cynomolgus monkey	18052	207	110
Rhesus monkey	33452	9634	118
Humans	17	18	73

The binding capacity of *Anti-CD25 mAb* and Simulect for human CD25 antigen were similar, with that of *Anti-CD25 mAb* being greater than that of Zenapax.
The species cross-reactivity to Cynomolgus and Rhesus PBMCs showed lower binding for *Anti-CD25 mAb* in comparison to human PBMCs, more evident in Rhesus species when the EC_{50s} were compared.
Due to the observed difference between Cynomolgus and Rhesus monkeys *Anti-CD25 mAb* cross-reactivity data, Cynomolgus monkeys can be considered suitable for use in toxicity safety studies.

2.5.4 Marmoset Monkey

Anti-CD25 mAb cross-reactivity was assessed on Marmoset monkey resting and activated PBMCs with the same test procedure and reagents used for Cynomolgus and Rhesus animal species.

No binding was recorded for the 3 products on resting human PBMCs, while on activated cells the binding was 10 times higher, confirming the data obtained in previous experiments (***Figures 18 and 19***).

Regarding Marmosets, no binding was obtained on resting PBMCs for the 3 products. On activated cells low binding activity was recorded in two out of four animals with *Anti-CD25 mAb* at 100 µg/mL only, while for Zenapax and Simulect relevant binding activity was recorded at both concentrations tested, 100 and 10 µg/mL (***Figures 20 and 21***).

Data sets demonstrate that healthy Marmoset monkey is not the relevant species to be used in safety studies since very poor cross-reactivity of *Anti-CD25 mAb* mAb to PBMCs was obtained.

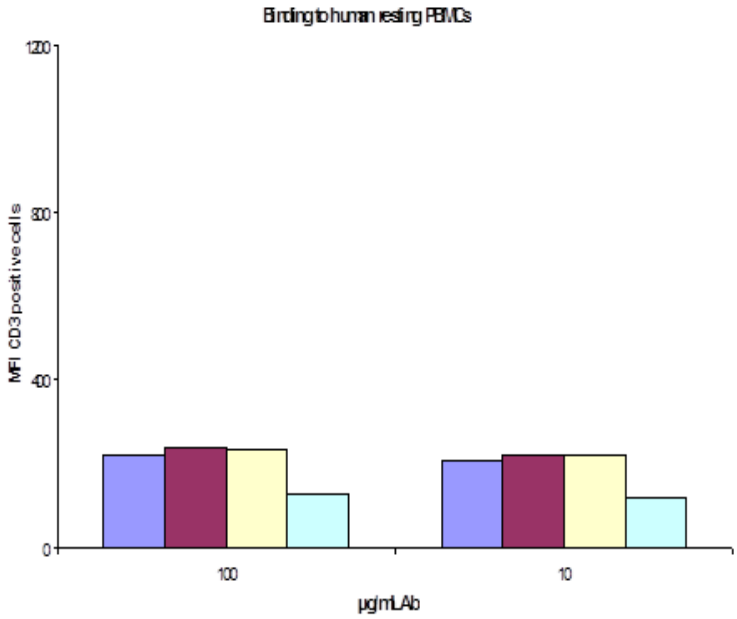


Fig. 18: *Cross reactivity evaluation in human resting PBMCs*
Cross reactivity evaluation of Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) and Zenapax competitor 2(purple) to human resting PBMCs (isotype control: light blue).

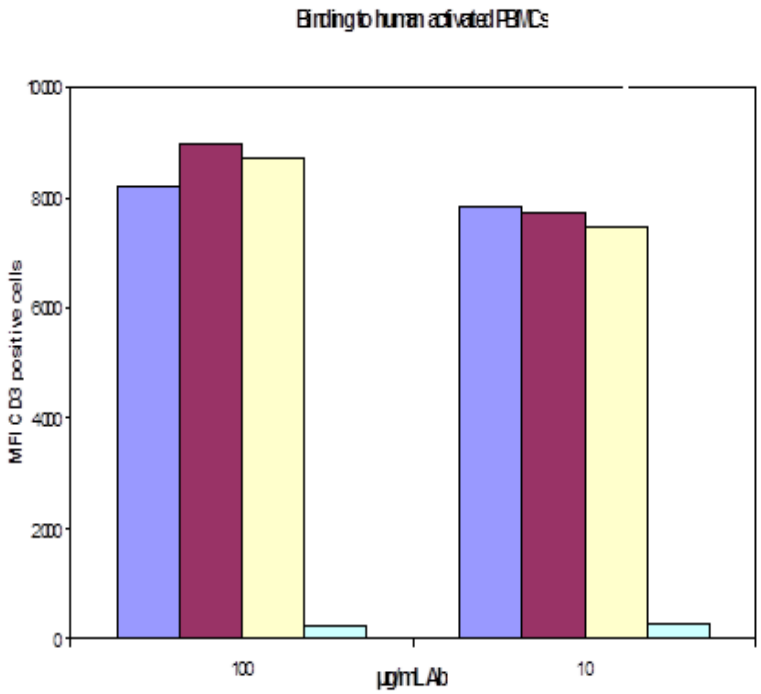


Fig. 19: *Cross reactivity evaluation in human <u>activated</u> PBMCs*
Cross reactivity evaluation of Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) and Zenapax competitor 2(purple) to human activated PBMCs (isotype control: light blue).

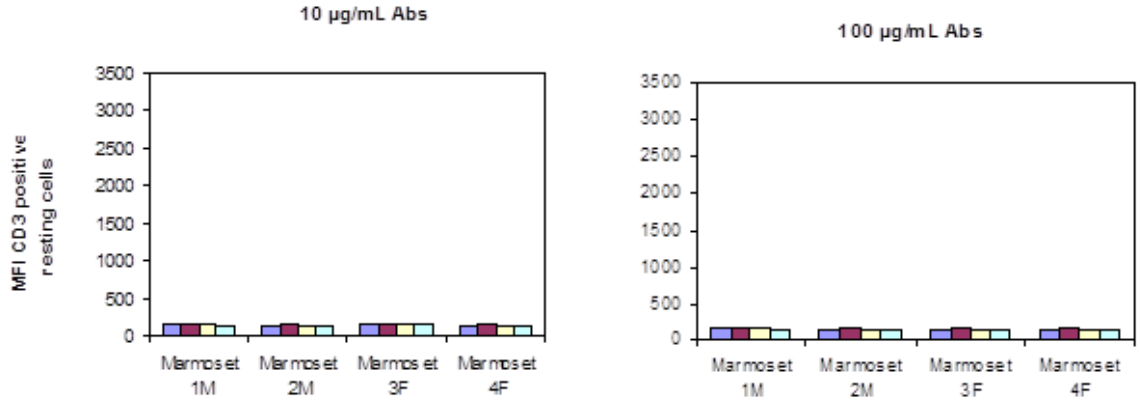


Fig. 20: *Cross reactivity evaluation in <u>resting</u> Marmoset Monkey PBMCs*
Cross-reactivity evaluation of Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) and Zenapax competitor 2(purple) to Marmoset monkey resting PBMCs (isotype control: light blue).

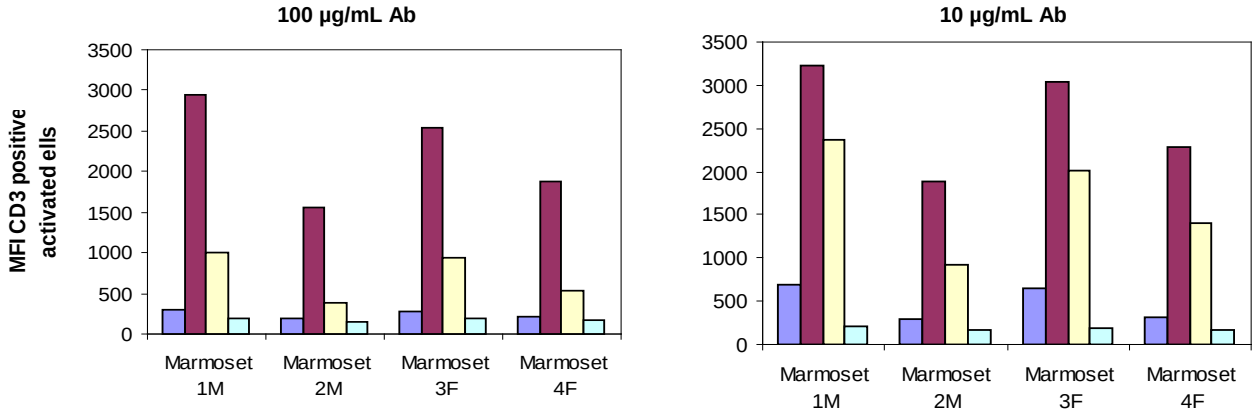


Fig. 21: *Cross reactivity evaluation in <u>activated</u> Marmoset Monkey PBMCs*
Cross-reactivity evaluation of Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) and Zenapax competitor

2(purple) to Marmoset monkey activated PBMCs (isotype control: light blue).

2.5.5 Minipig

Anti-CD25 mAb cross-reactivity was assessed on healthy Goettingen minipig resting and activated PBMCs.
Anti-CD25 mAb, commercial mAbs (Zenapax and Simulect) or isotype IgG control were used at the concentrations of 100 and 10 µg/mL on resting and activated PBMCs from four animals (2 males and 2 females) and one healthy human donor.

No binding was recorded for the 3 products on resting human PBMCs, while on activated cells the binding was 60 times higher confirming the data obtained in previous experiments (***Figures 22 and 23***).
No binding was obtained for the 3 products on minipig resting and activated PBMCs (***Figures 24 and 25***).

These data sets demonstrate that healthy minipigs are not the relevant species to be used in safety studies since no cross-reactivity of *Anti-CD25 mAb* to PBMCs was obtained.

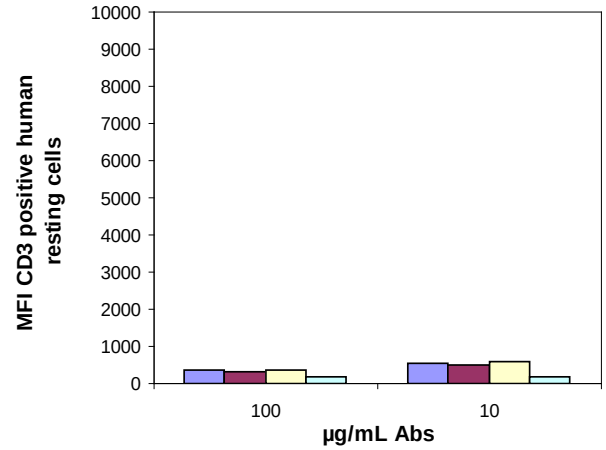


Fig. 22: *Cross reactivity evaluation in <u>resting</u> humanPBMCs*
Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) , Zenapax competitor 2(purple) and (isotype control: light blue).

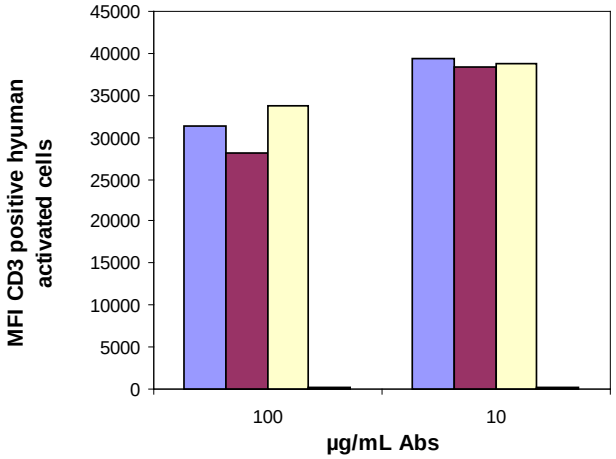


Fig. 23: *Cross reactivity evaluation in <u>activated</u> human PBMCs*
Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) , Zenapax competitor 2(purple) and (isotype control: light

blue).

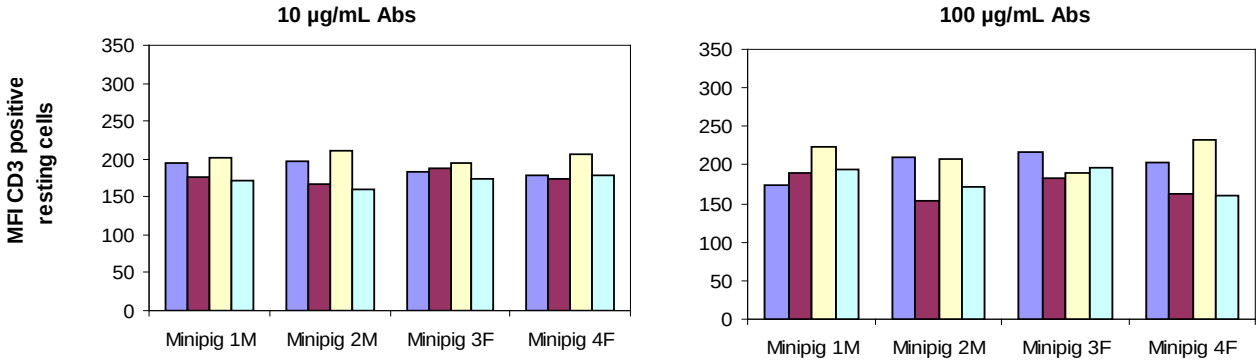


Fig. 24: *Cross reactivity evaluation in <u>resting</u> Minipig PBMCs*
Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) , Zenapax competitor 2(purple) and (isotype control: light blue).

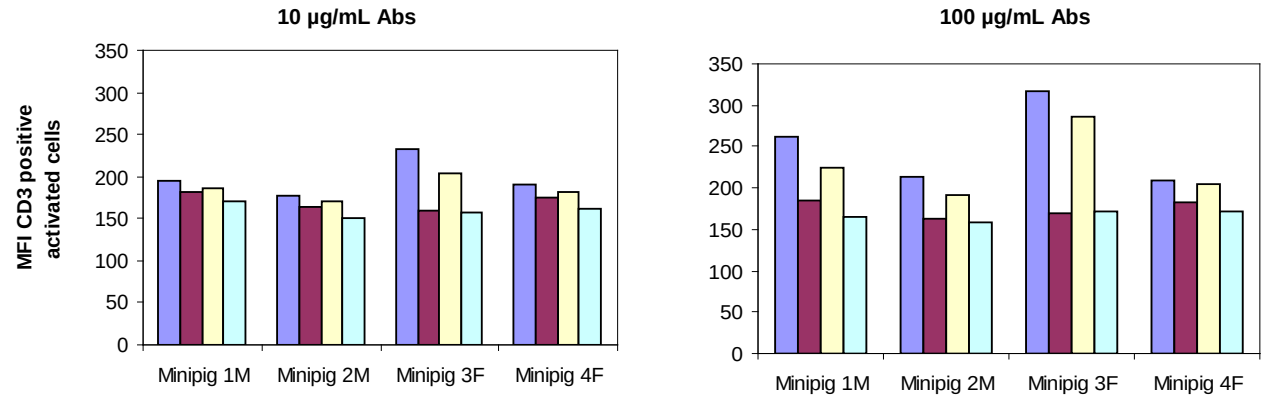


Fig. 25:*Cross reactivity evaluation in <u>activated</u> Minipig PBMCs*
Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) , Zenapax competitor 2(purple) and (isotype control: light blue).

2.5.6 Rabbit

Anti-CD25 mAb cross-reactivity was assessed on healthy New Zealand White rabbit resting and activated PBMCs.

Anti-CD25 mAb, commercial mAbs (Zenapax and Simulect) or isotype IgG control were used at the concentrations of 100 and 10 µg/mL on resting and activated PBMCs from four male animals and one healthy human donor.

No binding was recorded for the 3 products on resting human PBMCs, while on activated cells the binding was 60 times higher confirming the data obtained in previous experiments (***Figures 26 and 27)***.

No binding was obtained for Simulect (*competitor 1*) and Zenapax (*competitor 2*) on rabbit resting and activated PBMCs at both concentrations tested, while slightly higher MFI values only at 100 µg/mL of *Anti-CD25 mAb* was recorded and it was considered due to a aspecific

binding since occurred in either resting or activated rabbit PBMCs, and only at the higher concentration used (***Figures 28 and 29)***.

These data sets demonstrate that healthy rabbits are not the relevant species to be used in safety studies since no cross-reactivity of *Anti-CD25 mAb* to PBMCs was obtained.

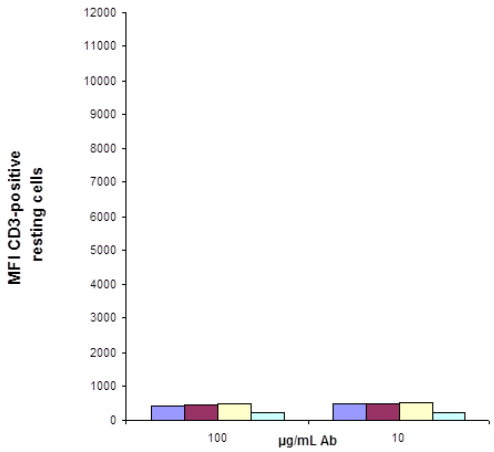


Fig. 26: *Cross-reactivity evaluation in <u>resting</u> human PBMCs*
Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) , Zenapax competitor 2(purple) and (isotype control: light blue).

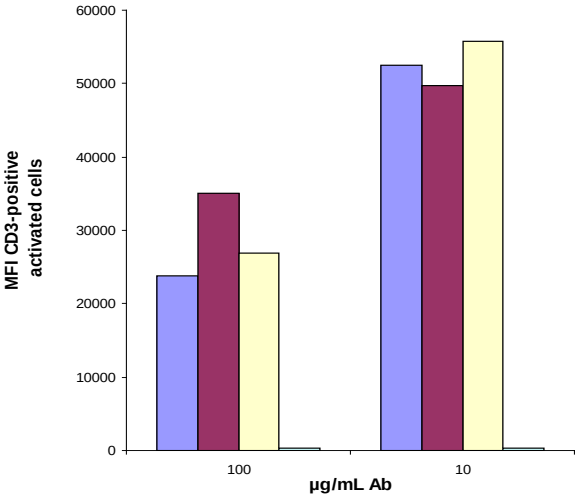


Fig. 27:*Cross-reactivity evaluation in <u>activated</u> human PBMCs*
Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) , Zenapax competitor 2(purple) and (isotype control: light blue).

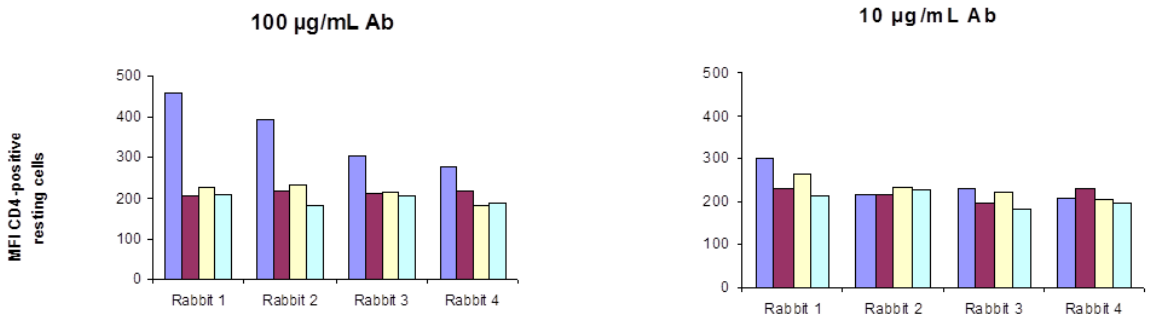


Fig. 28: *Cross-reactivity evaluation in resting rabbit PBMCs*
Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) , Zenapax competitor 2(purple) and (isotype control: light blue).

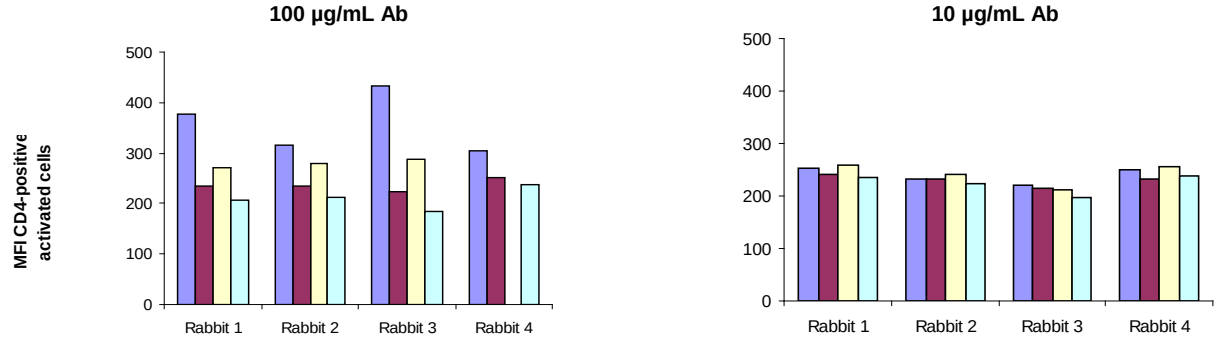


Fig. 29: *Cross-reactivity evaluation in activated rabbit PBMCs.*
Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) , Zenapax competitor 2(purple) and (isotype control: light blue).

2.5.7 Rat

Anti-CD25 mAb cross-reactivity was assessed on healthy Sprague-Dawley rats resting and activated PBMCs.

Anti-CD25 mAb, commercial mAbs (Zenapax and Simulect) or isotype IgG control were used at the concentrations of 100 and 10 µg/mL on resting and activated PBMCs from four animals (2 males and 2 females) and one healthy human donor.
These humans PBMCs have to be considered as control also for mice since these two animal species were tested together.
As expected, low and similar binding was recorded for the 3 products on resting human PBMCs, while on activated cells the binding was higher, confirming the data obtained in previous experiments (***Figures 30 and 31)***.

In rats, no binding was obtained for the 3 products on resting PBMCs.

On activated PBMCs low and similar binding was recorded for the 3 products at 100 µg/mL, while no binding was observed at the concentration of 10 µg/mL (***Figures 32 and 33)***.

These data sets demonstrate that healthy rats are not the relevant species to be used in safety

studies since no cross-reactivity of *Anti-CD25 mAb* to PBMCs was obtained.

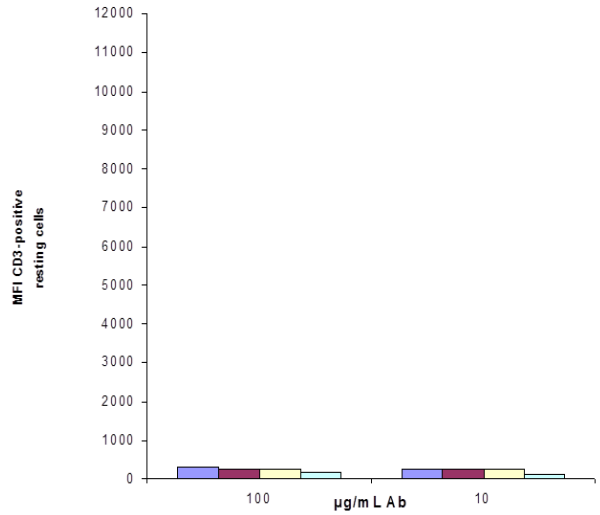


***Fig. 30**: Cross-reactivity evaluation in <u>resting</u> human PBMCs*
Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) , Zenapax competitor 2(purple) and (isotype control: light blue).

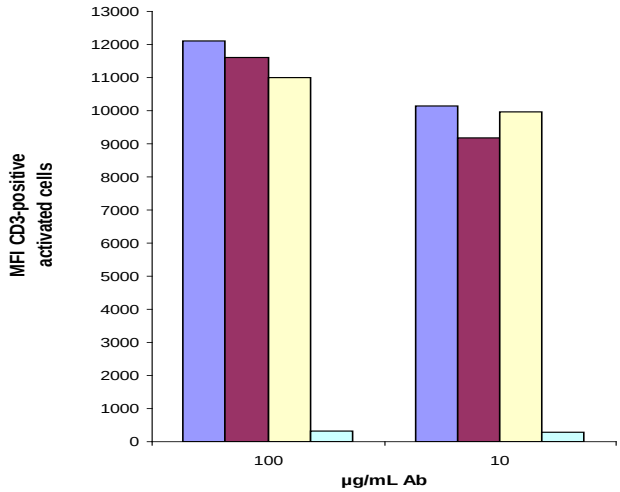


***Fig. 31:**Cross-reactivity evaluation in <u>activated</u> human PBMCs*
Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) , Zenapax competitor 2(purple) and (isotype control: light blue).

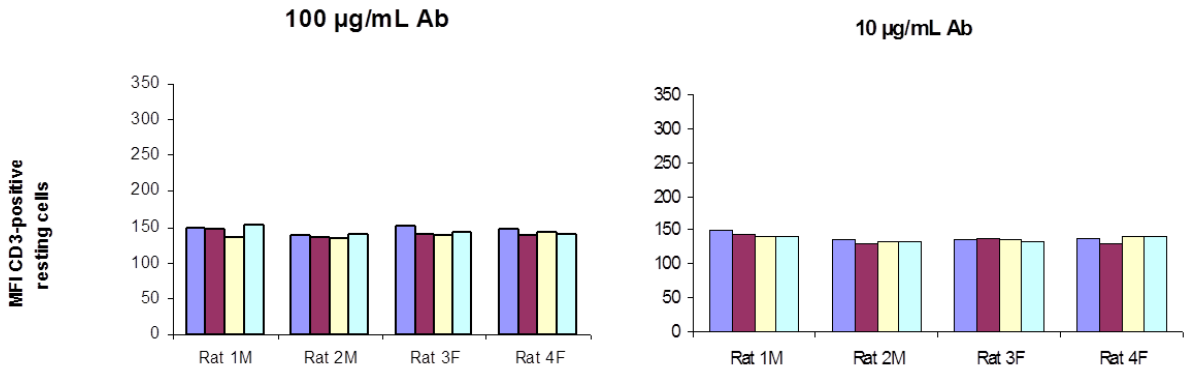


Fig. 32: *Cross-reactivity evaluation on <u>resting</u> rat PBMCs*
Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) , Zenapax competitor 2(purple) and (isotype control: light blue).

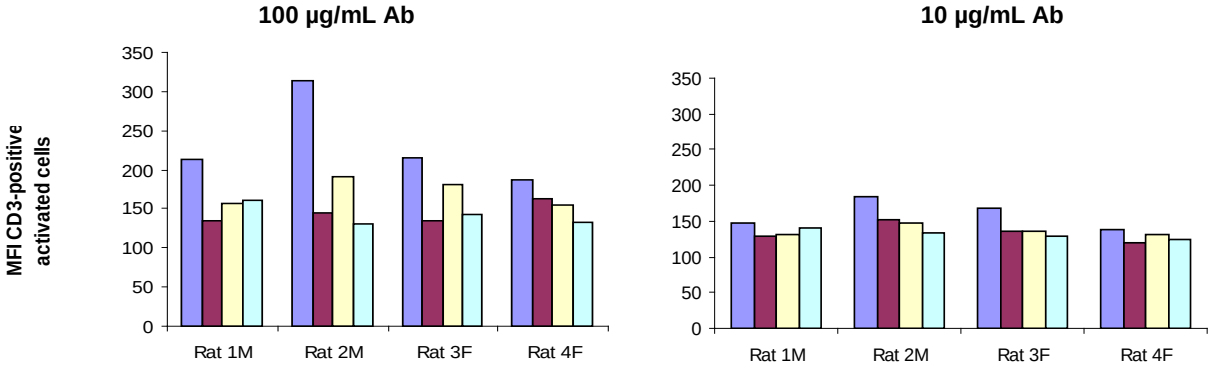


Fig. 33: *Cross-reactivity evaluation on <u>activated</u> rat PBMCs*
Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) , Zenapax competitor 2(purple) and (isotype control: light blue).

2.5.8 Mouse

Anti-CD25 mAb cross-reactivity was assessed on healthy CD1mice in three different assays.

In the first assay, *Anti-CD25 mAb*, commercial mAbs (Zenapax and Simulect) or isotype IgG control were used at the concentrations of 100 and 10 µg/mL on resting and activated PBMCs from four animals (2 males and 2 females) and one healthy human donor tested in parallel to mouse PBMCs (***Figures 34 and 35***).

No binding was obtained on resting PBMCs for the 3 products.

No binding was recorded for Simulect (*competitor 1*) and Zenapax (*competitor 2*), while a low binding was obtained with *Anti-CD25 mAb* at 100 µg/mL on activated PBMCs, even if a very poor CD25 expression was obtained on these cells.

Therefore, to better clarify the result obtained, a second assay was carried out using spleen lymphocytes instead of PBMCs (hard to obtain due to the limited volume of blood that can be drawn from each animal).

In this second assay (***Figure 36***), *Anti-CD25 mAb*, commercial mAbs (Zenapax and Simulect) or isotype IgG control were used at the concentrations of 100, 10, 1 and 0.1 µg/mL on resting and activated spleen lymphocytes from four animals (2 males and 2 females).

No binding was obtained on resting PBMCs for the 3 products.

As observed in the first assay, on activated PBMCs no binding was recorded for Simulect (*competitor 1*) and Zenapax (*competitor 2*), while a low binding was obtained with *Anti-CD25 mAb* at 100 µg/mL and again a poor CD25 expression was obtained.

Since the positive control used to evaluate the CD25 expression was unsatisfactory independently of the fluorocrome adopted (PE conjugated for PBMC and FITC conjugated for splenocytes) it was deemed necessary to optimize the procedure for mouse lymphocyte activation and to use it in a third assay (***Figure 37***) to define whether *Anti-CD25 mAb* is able to bind CD25 antigen expressed on mouse activated lymphocytes.

Two mitogens (PHA and ConA), different mitogen concentrations (PHA 5 µg/mL; ConA: 2.5, 5, 10 µg/mL), 3 differents activation times (24, 48, and 72 hours), two PE conjugated anti-mouse CD25 antibodies (BD Pharmingen and eBioscence) were tested on spleen cells.

Also in this third asssay *Anti-CD25 mAb*, commercial mAbs (Zenapax and Simulect) or isotype IgG control were used at the concentrations of 100, 10, 1 and 0.1 µg/mL on resting and activated spleen lymphocytes from four animals (2 males and 2 females).

As shown in ***Figure 38,*** no binding was obtained for the 3 products on CD1 mouse resting and activated spleen lymphocytes.

These data sets demonstrate that healthy CD1mice are not the relevant species to be used in safety studies since no cross-reactivity of *Anti-CD25 mAb* to lymphocytes was obtained.

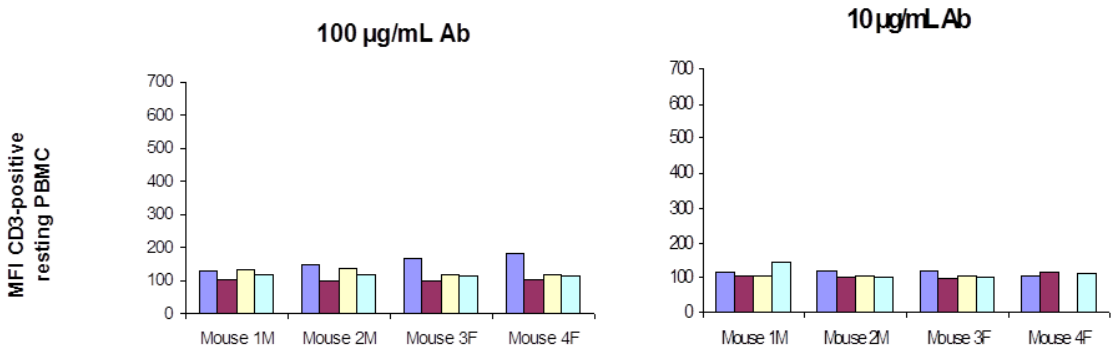


Fig. 34: *Cross-reactivity evaluation in <u>resting</u> mouse PBMCs*
Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) , Zenapax competitor 2(purple) and (isotype control: light blue).

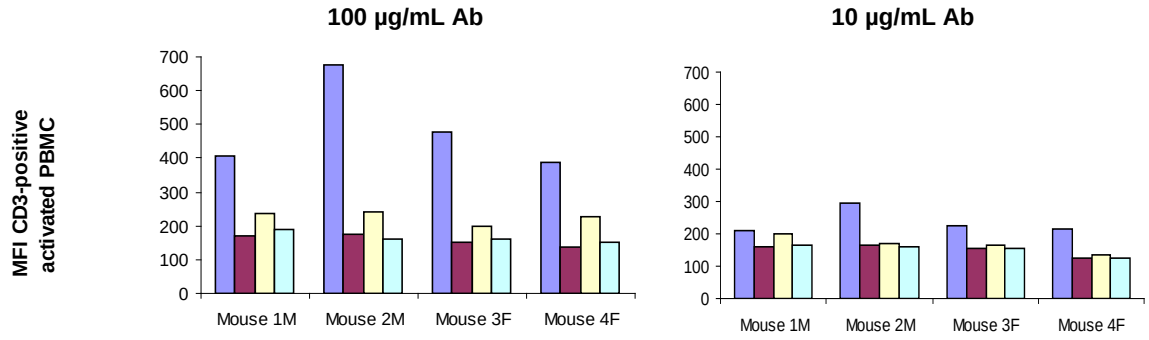


Fig. 35:*Cross-reactivity evaluation in <u>activated</u> mouse PBMCs*
Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) , Zenapax competitor 2(purple) and (isotype control: light blue).

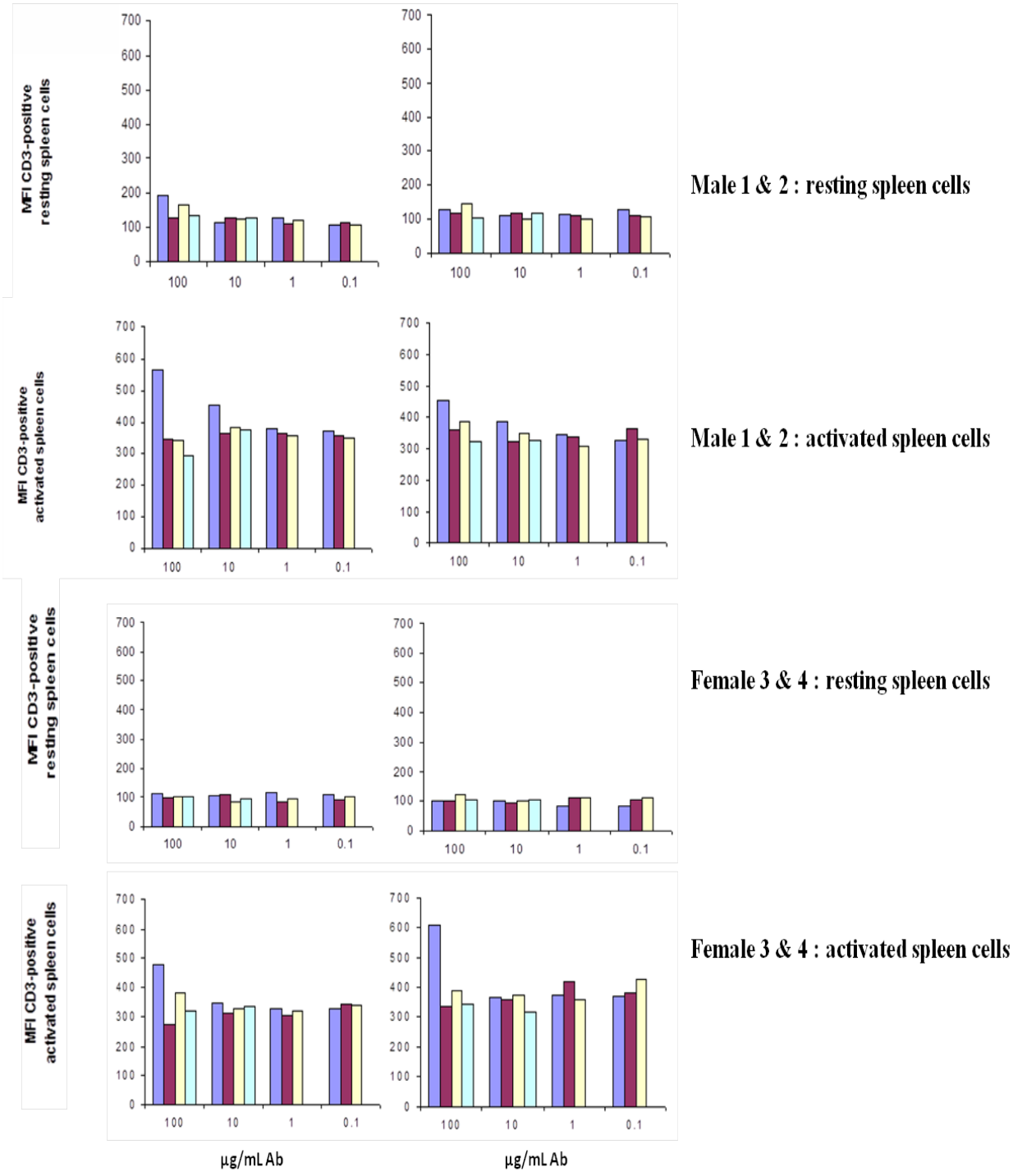
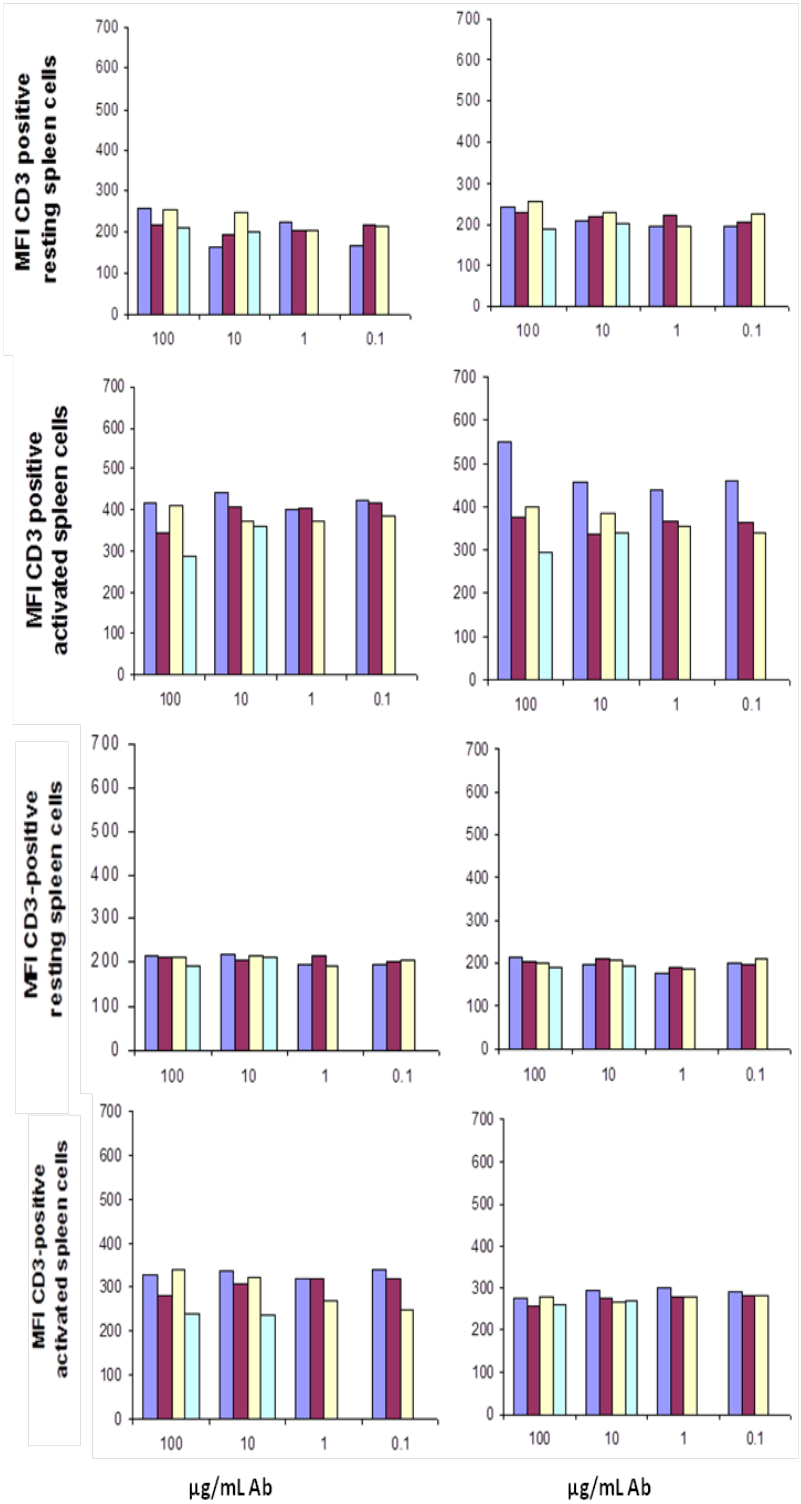


Fig. 36: *Cross-reactivity evaluation in <u>resting</u> and <u>activated</u> mouse spleen cells*
(FITC MFI of Abs binding on $CD3^+$ cells)
Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) , Zenapax competitor 2(purple) and (isotype control: light blue).



Male 1 & 2 : resting spleen cells

Male 1 & 2 : activated spleen cells

Female 3 & 4 : resting spleen cells

Female 3 & 4 : activated spleen cells

Fig. 37: *Cross-reactivity evaluation in resting and activated mouse spleen cells (FITC MFI of Abs binding on $CD3^+$ cells)*

Anti-CD25 mAb (violet), Simulect competitor 1(Yellow) , Zenapax competitor 2(purple) and (isotype control: light blue).

2.6 Cross- reactivity evaluation: conclusion

The aim of this investigation was to evaluate the cross-reactivity of *Anti-CD25 mAb* to PBMCs of the animal species most commonly used in toxicology investigations, in order to provide supportive data for selection of the most relevant one(s) to be used in safety studies.

The results obtained in Marmoset monkeys, Goettingen Mini-pigs, New Zealand white rabbits, Sprague Dawley rats and CD1 mice showed the absence of *Anti-CD25 mAb* cross-reactivity to PBMCs, and none of these species are therefore eligible.

Cross-reactivity with Rhesus PBMC was mild and lower than with Cynomolgus monkey PBMCs. Although the EC_{50} of *Anti-CD25 mAb* for PBMCs of Cynomolgus was higher than those determined for the marketed anti-CD25 mAbs, this was the only species where binding to CD25-bearing cells was shown at potentially relevant *in vivo* concentrations. Therefore the Cynomolgus monkey was considered relevant for the preclinical development program of *Anti-CD25 mAb*.

Based on the above-summarized results it was considered the Cynomolgus monkey be a suitable species to evaluate the safety, kinetics, dynamics and toxicity resulting from *Anti-CD25 mAb* administration.
It was also recognized that the extent of extrapolation of the monkey data to humans is somewhat limited by the lower capacity of *Anti-CD25 mAb* to bind monkey CD25.

The high dose levels used in the pre-clinical development were intended to overcome this potential issue. From the experience gained to date the % of CD25+ cells within the CD4+ T cell population in healthy Cynomolgus monkeys averaged 4% (range of 1.8 to 6.1%); human data either from HVs (healthy volunteers) and MS (Multiple Sclerosis) patients are being collected.

Data subsequently obtained in the *in vitro*/*ex-vivo* functional assays (e.g. inhibition of anti-CD3-stimulated PBMCs proliferation), coupled with the CD25 saturation data of the CD4+ T cells from the *in vivo* experiments collectively allowed to qualify the Cynomolgus monkey as a responsive animal species to *Anti-CD25 mAb.*

3. AIM OF THIS THESIS

Anti-CD25 mAb is a fully human IgG1 kappa monoclonal antibody (mAb) against the alpha subunit of the interleukin 2 receptor (IL2Rα, also known as CD25 or TAC antigen) of potential clinical interest in autoimmune diseases, including multiple sclerosis, uveitis, type 1 diabetes and psoriasis.

Anti-CD25 mAb acts mainly by inhibiting the proliferation of T cells and by consequence T cell clonal expansion and cytokine production. IL-2 is a potent immunomodulator whose major function is the activation of various cells of the immune system, i.e. T cells (including CD4+ CD25+ regulatory T cells), B cells, NK cells and macrophages, which express CD25 upon antigen stimulation.

The key to immunotherapeutic success with an anti-CD25 is to elicit the correct balance of effector and regulatory T cells. Nevertheless, the effects of an anti-CD25 antibody on the balance between pro-inflammatory T cells versus anti-inflammatory regulatory T cells are still unclear.

The potential advantages of *Anti-CD25 mAb* are the high affinity for human CD25 and the fact that it is a fully humanized *mAb*, potentially less immunogenic, leading to longer duration of therapeutic effect. It is difficult to foresee which subpopulation of CD25 cells will be the most inhibited by blocking the IL-2Rα as a consequence of *in vivo* administration of *Anti-CD25 mAb*. Such aspects were investigated in healthy non-human primates.

The purpose of the thesis is to assess the potential adverse effects resulting from the repeated administration of *Anti-CD25 mAb* in Cynomolgus monkeys, selected as the most relevant animal species for this preclinical safety study.

Anti-CD25 mAb was given by intravenous route (IV) at doses of 5, 25, 125 mg/kg or subcutaneously (SC) at a dose of 75 mg/Kg to 22 males and 22 females once a week for 5 consecutive weeks.

During the study general clinical observations, clinical pathology, CD25 expression, CD25 saturation and down-modulation on lymphocyte subsets, NK cell activity, cytokine release markers, T- cell proliferation and regulatory T cells number evaluation were determined.

One week after the last treatment 3 monkeys/sex/group were sacrificed for pathology investigations, while the remaining animals were subjected to a 28-week recovery period.

4. MATERIALS and METHODS

4.1 Test System

4.1.1 Characterization of the test system

Macaca fascicularis (Cynomolgus monkeys).

4.1.2 Justification for selection of the test system

The Cynomolgus monkey was chosen as non-rodent species since it has been found to be cross-reactive to the test compound.

4.1.3 Test system management

4.1.3.1 Supply, acceptance and selection

This study was performed on 22 male and 22 female bred Cynomolgus monkeys.

At the start of the study (day 1) the Cynomolgus monkeys were about 3-5 years old and the males weighed between 2.04 and 4.53 kg and the females between 1.83 and 3.09 kg.

Prior to their arrival at the test facility all monkeys had been quarantined and TB-tested with negative results. All animals were tested negative for Hepatitis B, Marburg and Ebola Viruses and were free from Salmonella/Shigella. Animals were also subjected to selected blood chemistry tests. During the pre-test period (3 weeks) preceding the start of treatment, all the animals were clinically examined by the test facility veterinarians to ascertain their fitness for the study.

Before the start of treatment all the animals were subjected to body weight and food consumption recordings, ophthalmological examinations, ECG recordings, hematological and blood chemistry tests and urinalysis.

The test-eligible animals were then randomly allocated to the different dose groups and the identification number of each animal was marked on its cage card.

4.1.3.2 Housing

The monkeys were caged singly in sheet stainless steel cages with front wall and bottom in stainless steel grill. The cages were hung on metal racks. The cages were housed in an air conditioned (24°C±2°C) room, with 15 – 20 air changes per hour, relative humidity of 55% ± 15, artificial lighting with a circadian cycle of 12 hours (7 a.m. - 7 p.m.).

4.1.3.3 Diet and water supply

A diet coded "3KE25 GLP top certificate", produced for Charles River Italia by its licensee Mucedola S.r.l., Settimo Milanese, analyzed for nutrients, was used.

With the diet, the producer supplied a Certificate of analysis in which it declared that the presence and the levels of contaminants in the diet were within the limits proposed by the EPA-TSCA (44FR:44053- 44093, July 26, 1979).

The animal feed is re-analyzed at least twice a year for bacterial contamination. The ration was distributed once a day in the total amount of about 200 g/monkey. Fruit or vegetables were distributed twice a day. Filtered water was distributed by means of an automatic watering valve system. The drinking water offered to the animals "ad libitum" came from the Ivrea regional water main.

Periodically the drinking water is analyzed for microbiologic count, heavy metals, other contaminants (e.g. solvents, pesticides) and other chemical and physical characteristics.
The acceptance limits of quality of the drinking water were those defined in EEC Directive 80/778. Contaminants that might interfere with the objectives of this study were not expected to be present either in the diet or in the drinking water.

4.2 Experymental Design

4.2.1 Treatment schedule

4.2.1.1 Doses, Volume, concentration

The animals were randomly allocated to 4 dose groups plus 1 control group according to the scheme below:

Group	***Doses*** ***(mg /kg/ week)*** ***[administr.Route]***	***Volume of administr.*** ***(ml/Kg)***	***Concentr. In vehicle*** ***(mg/ml)***	***No. of males***	***No. of females***	***Group color identification***
1	0 (vehicle) [IV]	2	0	4	4	white
2	5 [IV]	2	2.5	4	4	yellow
3	25 [IV]	2	12.5	4	4	green
4	125 [IV]	2	62.5	5	5	red
5	75 [SC]	2	37.5	5	5	blue

The administration volume was 2 ml/kg and was adjusted on the basis of the most recently recorded body weight (individual values). During the study general clinical observations, ECG recordings, ophthalmoscopy, body temperature and body weight and food consumption measurements were carried out.

Laboratory investigations (hematology, blood chemistry and urinalysis) were also performed.
Samples for toxicokinetics purposes and additional evaluations were also collected.

One week after the last treatment 3 monkeys/sex/group were sacrificed for pathology investigations, while the remaining animals were subjected to a 29-week recovery period and then sacrificed.

4.2.1.2 Administration regimen

The test article was administered once a week.

4.2.1.3 Administration route

The intravenous [IV] and subcutaneous [SC] routes are the anticipated therapeutic routes.

Intravenous route, injection in the right saphena vein.
Subcutaneous route, in the subcutis of the right leg.

4.2.2 Test system identification and grouping criteria

The 44 test- eligible monkeys were randomly allocated to the experimental groups, as detailed below.

All monkeys were identified using the last digits of the number tattooed by the Supplier.

Group 1 (white)	**0 mg/kg/week (vehicle)**
	Male No.s: 0311051 - 0311353 - 0312045 - 0406025(§)
	Females No.s: 0109042 - 0212152 - 0302670 - 0309164 (§)

Group 2 (yellow)	**5 mg/kg/week (IV)**
	Male No.s: 0212101 - 0309087 - 0311021 - 0401043 (§)
	Females No.s 0312224 - 0312342 - 0401126 - 0401208 (§)

Group 3 (green)	**25 mg/kg/week (IV)**
	Males No.s 0208097 - 0209075 - 0310153 - 0402027 (§)
	Females No.s 0109110 - 0304172 - 0312398 - 0312490 (§)

Group 4 (red)	**125 mg/kg/week (IV)**
	Males No.s 10103 - 0308363 - 0309073 - 0310411 (§) - 0404015 (§)
	Females No.s 0306520 - 0307610 - 0308074 - 0312210 (§) - 0402134 (§)

Group 5 (blue)	**75 mg/kg/week (SC)**
	Males No.s 0202205 - 0210253 - 0309365 - 0312003 (§) - 0401037 (§)
	Females No.s 0108190 - 0302688 - 0303226 - 0307140 (§) -0401222 (§)

§: animals subjected to recovery period

4.2.3 Experimental procedures

4.2.3.1 Clinical observation

4.2.3.1.1 Mortality

Animals were inspected twice a day for mortality (early in the morning and again in the afternoon).

4.2.3.1.2 Clinical Signs

All monkeys were monitored for physical appearance, behaviour and general clinical signs before and after each administration. Moreover, frequent inspections were done after each dosing.

During the recovery period the animals were inspected twice, early in the morning and again in the afternoon.

Individual clinical observations were made in order to detect abnormalities related to the involvement of CNS, Tegumental, Digestive, Musculoskeletal, Respiratory and Genitourinary apparatus.

All signs of ill health, together with any behavioural change or reaction to treatment, were recorded for each animal. Dated and signed records of clinical signs were kept on clinical history sheets for individual animals.

4.2.3.1.3 Body weight, food consumption, rectal temperature

All the animals were weighed twice prior to the beginning of treatment and then weekly during the dosing and recovery periods.

Food consumption was measured once pre-trial and during the treatment and recovery periods, when the body weight was recorded. At each of the scheduled times, the residual feed from that offered (2000 g gross weight, equivalent to about 1400 g net weight, subdivided into daily portions of about 200 g) was weighed; consumption of food was calculated and expressed as g/animal/day.

Rectal temperature was measured twice pre-trial and then before and at 2, 6 and 24 hours after each treatment. Measurements were always performed before blood sampling.

4.2.3.1.4 Ophthalmological examinations

Both eyes of all animals assigned to the study were examined once pre-trial. The same examination was repeated at the end of treatment (week 5) and during weeks 7 and 26/27 of the recovery period.

Eyes were initially examined by macroscopic observation (naked eye); then conjunctiva, cornea, anterior chamber, iris, lens, vitreous body and fundus were examined with an Omega 100 indirect ophthalmoscope (Heine), after instillation of 1% Tropicamide (Visumidriatic - Visufarma).

4.2.3.1.5 Electrocardiography and heart rate

ECG tracings were recorded on the animals according to Bailey's lead system by a three-channel Bosch EKG 506 D electrocardiograph (amplification automatically adjusted according to R wave amplitude).

Electrocardiogram spot tracings were recorded once pre-study and then on 2 animals/sex of groups 1, 2 and 3 (those with the highest identification number), and on 3 animals/sex of groups 4 and 5 (those with the highest identification number).
Heart rate was calculated on the basis of systolic complexes occurring in an ECG tracing within a period of 6 seconds.

During the ECG recordings the animals were kept in right lateral recumbency and held with hind and forelegs at right angles to the body and parallel to each other by an assistant. Insulated alligator clips were used as electrodes.

Electrocardiogram tracings were recorded at the following times:

- Before the start of treatment (day -10 for males and day -7 for females)
- On the animals treated by intravenous route:
    - 1 hour after the treatment performed in weeks 2 and 4 and in weeks 8 and 27/28 of recovery.
- On the animals treated by subcutaneous route:
    - 3 hours after the treatment performed in weeks 2 and 4 and in weeks 8and 27/28 of recovery.

4.2.3.1.6 Laboratory investigations (Hematology, blood chemistry and urinalysis)

In order to permit urine collection, the monkeys were kept in metabolism cages for about 16 hours without food and water, after having received 20 mL/kg of tap water by gavage as water load, before the scheduled analysis day.

Blood samples (3.0 mL/each sampling) were drawn in the morning from a cephalic vein of each fasted animal. Hematological examinations, blood chemistry tests (including IgG and IgM, Fibrinogen, alpha 1 antitrypsin (α1 AT), alpha 1 acid glycoprotein (α1 AGP), C-reactive protein (CRP) tests and urinalyses were performed in all the animals following the scheme below:

Period	Week	Test
Pre- trial	- 2	Hematology (+Fibrinogen) Blood Chemistry (+ AT, AGP, CRP, IgG and IgM), urinalyses
Treatment	1	Fibrinogen, APT, AGP, CRP.
Treatment	3	Fibrinogen, APT, AGP, CRP.
Treatment	5	Fibrinogen, APT, AGP, CRP.
Treatment	6	Hematology (+Fibrinogen) Blood Chemistry (+ AT, G AGP, CRP, IgG and IgM), urinalyses
Recovery	8	Hematology (+Fibrinogen) Blood Chemistry (+ AT, AGP CRP, IgG and IgM), urinalyses
Recovery	13	Hematology (+Fibrinogen) Blood Chemistry (+ AT, AGP, CRP, IgG and IgM), urinalyses
Recovery	28	Hematology (+Fibrinogen) Blood Chemistry (+ AT, AGP, CRP, IgG and IgM), urinalyses

Legend: AGP = Alpha-1-acid glycoprotein, CRP = C-reactive protein, AT = alfa-1-antitrypsin

4.2.3.1.7 Pathology (Necropsy and Histopathology)

At the end of the treatment (week 5) and recovery (week 28) periods each animal was fasted overnight (about 16 hours); the fasted body weight was recorded before sacrifice.

The animals were sacrificed after being anesthetized with an i.v. injection of an overdosage of barbiturate (50 mg/kg). Each anesthetized animal was bled to death through the femoral arteries and subjected to a complete necropsy.

Organs and tissues were collected and the organs weighed in all animals as well as the fixative(s) used. Histological examination was performed on all animals sacrificed after 4 weeks of treatment and in the animals which underwent recovery.
Individual organ weight/fasted body weight ratios were calculated. The organs to be examined were embedded in paraffin blocks, sectioned and stained with hematoxylin and eosin. Bone tissues were decalcified before being embedded.

4.2.3.1.8 Toxicokinetics

Blood samples (about 0.5 mL/sample) were collected for toxicokinetic purposes from a cephalic vein of all animals following the schedule below:

- *In monkeys treated by intravenous route:*

- Basal time point =0
- After the 1st treatment: 5 minutes, 30 minutes, 1, 2, 3, 6, 24, 96 and 168 hours.
- From the 2nd to the 4th treatment: before treatment and after 30 minutes.
- At the 5th treatment: before treatment and after 0, 5 minutes, 30 minutes, 1, 2, 3, 6, 24, 96 and 168 hours.
- During recovery period: once a week during W1, W2, W3, W4, W5, W6, W7, W10, W14, W23 W26 and W28 for the female, and during W1, W2, W4, W5, W6, W7, W8, W11, W14, W20, W27 and W28 for the male.

- *In monkeys treated by subcutaneous route:*

- Basal time point =0
- After the 1st treatment: 30 minutes, 2, 6, 24, 96 and 168 hours.
- From the 2nd to the 4th treatment: 4 hours.
- At the 5th treatment: 0, 30 minutes, 2, 6, 24 and 96 hours.
- During recovery period: once a week during W1, W2, W3, W4, W5, W6, W7, W10, W14, W23 W26 and W28 for the female, and during W1, W2, W4, W5, W6, W7, W8, W11, W14, W20, W27 and W28 for the male.

The blood samples collected were allowed to clot for 50-60 minutes at room temperature. The clot was spun down by centrifugation at 3000 rpm for 15 minutes and the supernatant was collected and divided into two aliquots, which were stored frozen at –80°C until analysis.

Serum samples were analyzed for Anti- CD25 quantification using the validated ELISA method.

4.2.3.1.9 PD markers: leukocyte and lymphocyte subsets; CD25 expression, saturation and down-modulation on lymphocyte subsets.

Whole-blood samples were obtained for PD analysis at the following time points:

- Pre- treatment - All animals (D1)
- After 1st treatment - 3 h after treatment (D1), in monkeys treated by IV route
  - 6 h after treatment (D1), in monkeys treated by SC route
- From 2nd to 5th administration - All animals on the day before treatment (D7,D14, D21, D28)
- Recovery period - all animals, in weeks 2, 4, 6, 8, 11, 14, 18, 23, 29

At the time points listed above, the following parameters were recorded:

- *Total leukocytes and leukocyte subsets* (lymphocytes and neutrophils, absolute count, monocyte percentage and absolute counts), as determined by hematological analysis.

- *Lymphocyte subsets* (percentage and absolute values) of total T-cells (CD3+), helper T-cells (CD3+CD4+), cytotoxic T-cells (CD3+CD4-), B-cells (CD3-CD20+) and Natural Killer (NK) cells (CD3-CD16+), determined by flow cytometry (FACS) analysis.

- *CD25 expression/saturation/down-modulation* (cell percentage) on the following lymphocyte subsets: total T-cells, helper T-cells, cytotoxic T-cells, as determined by FACS analysis.

- *CD25 expression/saturation* was determined using anti-human CD25 PE-conjugated monoclonal antibody (mAb) (clone ACT-1; Dako Cytomation). Clone ACT-1 binds to an epitope similar to that used by Anti- CD25 and therefore binds only to free CD25 antigen.

- *CD25 down-modulation* was determined using anti-human CD25 PE-conjugated mAb (clone 7G7B6; Ancell). Clone 7G7B6 binds to a CD25 epitope different from that bound by Anti-CD25 and therefore it can bind to all the CD25 molecules expressed on the cell surface. This antibody recognizes the human IL-2 receptor but binds to an epitope distinct from that recognised by either IL- 2 or Anti-CD25 mAb.
  Therefore the ability of Anti-CD25 to bind to and saturate IL-2Rα sites on lymphocytes was assessed indirectly by loss of anti-CD25/ACT-1 binding relative to 7G7/B6 binding.

- *Anti-CD25 mAb (test item) bound to the cell surface*, as determined by FACS analysis using an anti-human IgG PE-conjugated mAb; this parameter was recorded as follows:
  - in all animals undergoing recovery starting from week 4 or 2 of withdrawal for males and females respectively, to monitor the reduction in Anti-CD25 and, consequently CD25 desaturation.
  - in some animals sacrificed at the end of treatment at various analytical time points.

Low monocyte counts (percentage and absolute values) were recorded in some animals at various sampling times. These monocyte values were flagged as outliers and, for the statistical evaluation, they were replaced with the mean value of all animals of the same group at the time point considered.

Immunophenotyping analysis and CD25 expression/saturation/down-modulation were performed on peripheral blood collected in EDTA (0.5 mL blood/sampling time) and analyzed by flow cytometry using direct immunofluorescence and lyse-no-wash techniques.
Commercially available human mAbs that crossreact with Cynomolgus monkey antigens were

used at the concentrations recommended by the manufacturer or the best applicable dilutions tested in a preliminary study.

ANTIBODY PANEL

MoAb	Clone	Source
Mouse IgG1k PE isotype control	MOPC-21	BD Pharmingen
Mouse IgG2a PE isotype control	G155-178	BD Pharmingen
Mouse anti-human CD3-APC-Cy7	SP34-2	BD Pharmingen
Mouse anti-human CD4-APC	L200	BD Pharmingen
Mouse anti-human CD20-FITC	2H7	BD Pharmingen
Anti-human CD16-FITC	3G8	BD Pharmingen
Mouse anti-human CD45-PerCP	D058/1283	BD Pharmingen
Mouse anti-human CD25-PE (IgG1k)	ACT-1	Dako Cytomation
Mouse anti-human CD25-PE (IgG2ak)	7G7B6	Ancell
Anti-human IgG Fcγ fragment specific-PE	N.A.	eBioscience

Lymphocyte subsets and CD25 expression/saturation/down-modulation were detected with the following combinations of markers diluted with PBS/0.1% BSA and appropriately pooled:

IMMUNOPHENOTYPING – CD25 EXPRESSION/SATURATION

Pool	Markers	Cell Population
A	CD45PerCP/CD4APC/CD20FITC/CD3APCCy7/ CD25PE (Dako)	CD3+: TotalT lymphocytes CD3+CD4+: T helper cells CD3+CD4-: T cytotoxic cells CD3-CD20+: Total B cells CD3+CD25+ CD3+CD4+CD25+ CD3+CD4-CD25+
B	CD45PerCP/CD16FITC/CD3APC-Cy7	CD3-CD16+: NK cells

CD25 DOWN-MODULATION

Pool	Markers	Cell Population
A	CD45PerCP/CD4APC/CD20FITC/CD3APCCy7/ CD25PE (Ancell)	CD3+CD25+ CD3+CD4+CD25+ CD3+CD4-CD25+

For each individual sample 25 or 15 μL of antibody pool A and pool B, respectively, were added to 25 μL of whole blood.

Isotype controls in each combination were run to assess the presence of non-specific staining and to set quadrants for dot plot analysis of either CD25 expression (Dako mAb) or CD25 down-modulation (Ancell mAb). They were used for staining the blood samples from the first animal (in numerical order) in each group, and were dispensed in the tubes as follows:

Tube 1: mouse IgG1k PE, anti-CD3 APC-Cy7, anti-CD4 APC, anti-CD20 FITC, anti-CD45 PerCP

Tube 2: mouse IgG2a PE, anti-CD3 APC-Cy7, anti-CD4 APC, anti-CD20 FITC, anti-CD45 PerCP

The tubes were mixed and incubated in the dark for 15 min at room temperature.

1 mL of ammonium chloride lysing solution was added and, after mixing, the tubes were kept in the dark for 15 min. at room temperature. The tubes were run on a FACSCanto instrument within one hour of the end of preparation.

A FACSCanto flow cytometer (BD Biosciences) equipped with 488 nm and 633 nm laser lines and FACSDiva software (BD Biosciences) was used for acquisition and analysis of all samples. BD FACS 7-color setup beads (BD Biosciences) were run in order to check proper instrument setting and detector linearity.

The lymphocyte acquisition gate was set on CD45 fluorescence/side scatter plot and a minimum of 5000 events were acquired within the lymphocyte gate.
Each lymphocyte subpopulation was analyzed and referred to a lymphocyte gate set on forward/side scatter parameters, and expressed as number of events as well as percentage of cells within the lymphocyte gate.

Estimates of the absolute cell counts of lymphocyte subsets were obtained using dual-platform counting technologies, calculated from the percentage of cells in each subset obtained by flow cytometry and the absolute lymphocyte cell count obtained by automated hematology analyzer.

CD25 expression/down-modulation on Total, CD4+ and CD8+ T-cells, was referred to the appropriate lymphocyte subpopulation and expressed as percentage of CD25-positive cells.

Statistical analysis was performed in GraphPad Prism considering males and females together.

The following parameters were analyzed:

- CD25 expression, saturation and down modulation on CD4+ T-cells (cells %);
- lymphocyte subsets and monocytes (absolute counts);
- Hematological parameters: lymphocytes, neutrophils and white blood cells (WBC) (cells/µL of blood).

For each parameter, data obtained at the different time points were compared with pre-treatment values; a control group of untreated animals was included.

A repeated measurements one-way ANOVA per group, across time, for each parameter was used. It was then followed by a post-hoc test (Dunnett's multiple comparison test of each time point against the pre-dose value).

Statistical analysis of the parameters obtained during the recovery period was not performed due to the limited number of animals/group: 1 male + 1 female in groups 1, 2 and 3; 2 males + 2 females in groups 4 and 5.

4.2.3.1.10 Other Biomarkers

4.2.3.1.10.1 Natural killer (NK) cell activity

NK activity was measured by the ability of effector cells (PBMC) to kill the target cells (human K562 cell line).

Heparinized blood samples were obtained from the blood collected through the saphena vein from all animals at the following time point: pre-dose, at the end of treatment (on week 5) and at

three timepoints during the recovery period (in weeks 13, 25 and 28).

Orpegen Pharma NKTEST kit for the quantification of the cytotoxic activity of natural killer cells was used following the manufacturer's instructions.

PBMCs were separated by Ficoll procedure and incubated with pre-stained (green fluorescence) K562 target cells for 2 hours at an effector/target ratio of 25:1.
Each sample was prepared in triplicate.

Moreover, positive control samples were prepared adding IL2 prior to incubation and control samples with target cells alone to measure spontaneous cell death (6 replicates).
Cells were stained for 5 min with a DNA staining solution, to label killed cells, and immediately analyzed with FACSCanto (FACSDiva software).

Target cells were gated and the percentage of dead target cells was obtained.
Results were expressed as % of NK specific activity, determined by subtracting the percentage of dead cells in the control tubes from the percentage of killed target cells in the test samples.

4.2.3.1.10.2 CRS MARKERS (TNFα, IL-6, IFNγ, IL-1β, IL-2, IL-4, IL-10, IL-8, MCP-1)

In order to perform cytokine profiling investigations, blood samples were collected from animals at the following time points:

Group	Route	No. of animal	Dose mg/ Kg	Dosage regimen		Time Points						
						Day 0	Day 1			Day 28		
1	IV	4M+4F	Vehicle	Once a week		Predose	2h	6h	24h	2h	6h	24h
2	IV	4M+4F	5	Once a week		Predose	2h	6h	24h	2h	6h	24h
3	IV	4M+4F	25	Once a week		Predose	2h	6h	24h	2h	6h	24h
4	IV	5M+5F	125	Once a week		Predose	2h	6h	24h	2h	6h	24h
5	SC	5M+5F	75	Once a week		Predose	2h	6h	24h	2h	6h	24h

At each selected time point 0.5 mL of blood were collected in tubes with Na-Citrate as anticoagulant and centrifuged at 2500g at +4°C for 10 min. The supernatant (plasma) was collected and divided into three aliquots, of at least 60 µL each, which were stored frozen at -80°C until analysis.

The Luminex100 system, a flow cytometer with two lasers able to perform the simultaneous detection of several cytokines/chemokines, was used in this study.

The principle of the test is based on a regular sandwich ELISA where the primary antibody is coated onto a fluorescent polystyrene bead.
These beads can be detected and sorted univocally by the first laser (635 nm) while the fluorochrome reporter (phycoerythrin), can be quantified by the second laser (523 nm). Different bead sets were combined in a single well to allow multiplex assays using a small amount of biological sample.

The concentrations of the following cytokines/chemokines were determined:

A) IFNγ, IL-2, IL-4, IL-10, TNFα, MCP-1, IL8 (kit for monkey cytokines/chemokines);
B) IL-6 kit for human IL-6);
C) IL1β (kit for monkey IL-1β).

Two commercial Luminex kits (Monkey Cytokines 5-plex – Cat # LPC0001 and Monkey Chemokines 5-plex – Cat # LPC0002, BioSource International Inc., Camarillo, CA) were mixed and used with their own reagents, buffers and standards, according to the leaflet (***Figure 38***) upon an optimization phase.

A) The two kits included 10 analytes (IFNγ, IL-2, IL-4, IL-10, TNFα, MCP-1, MIP-1α, MIP-1β, RANTES, IL8) but MIP-1α, MIP-1β and RANTES were not analyzed since of no interest for this study.

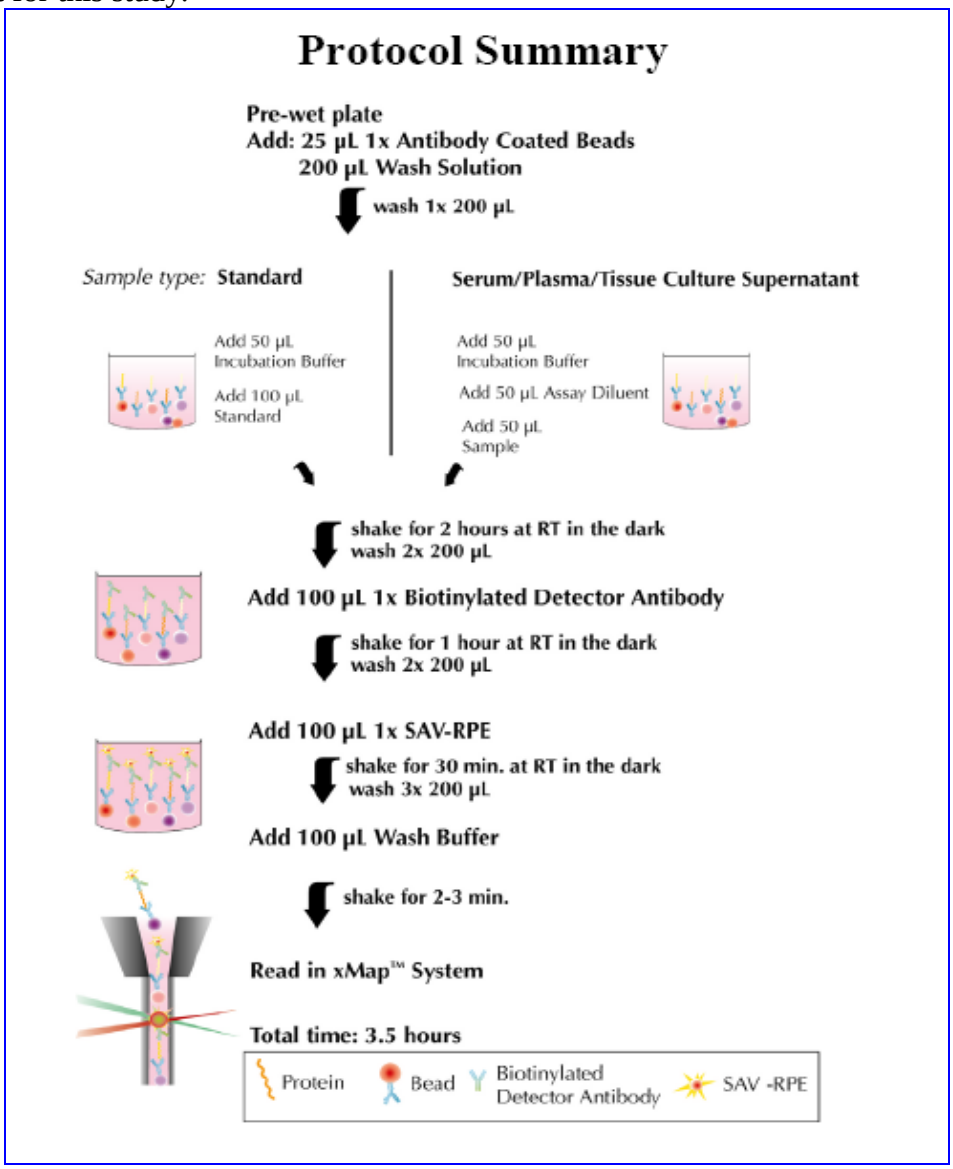


Fig. 38: *Determination of IFNγ, IL-2, IL-4, IL-10, TNFα, MCP-1, IL8*

Washing steps were performed in 1.2 µm filter membrane 96-well microtiter plates (Millipore Corp., Billerica, MA) with a multiscreen vacuum manifold for 96-well plates (Millipore). Incubations were done on a plate shaker Mini OrbitalShaker (Bellco Glass, In) at room temperature. The plates were read using the above-mentioned Luminex100 System (Luminex Corp., Austin, TX).

B) A commercial Luminex kit (Human IL-6 single-plex – Cat # 171-B10719, Bio-Rad Laboratories, Hercules, CA) was used with its own reagents, buffers and standards, according to the leaflet (see figure below) upon an optimization phase. In particular, the

standard curve was run in a pool of monkey plasma (Na-Citrate) instead of using the buffer provided by the vendor.

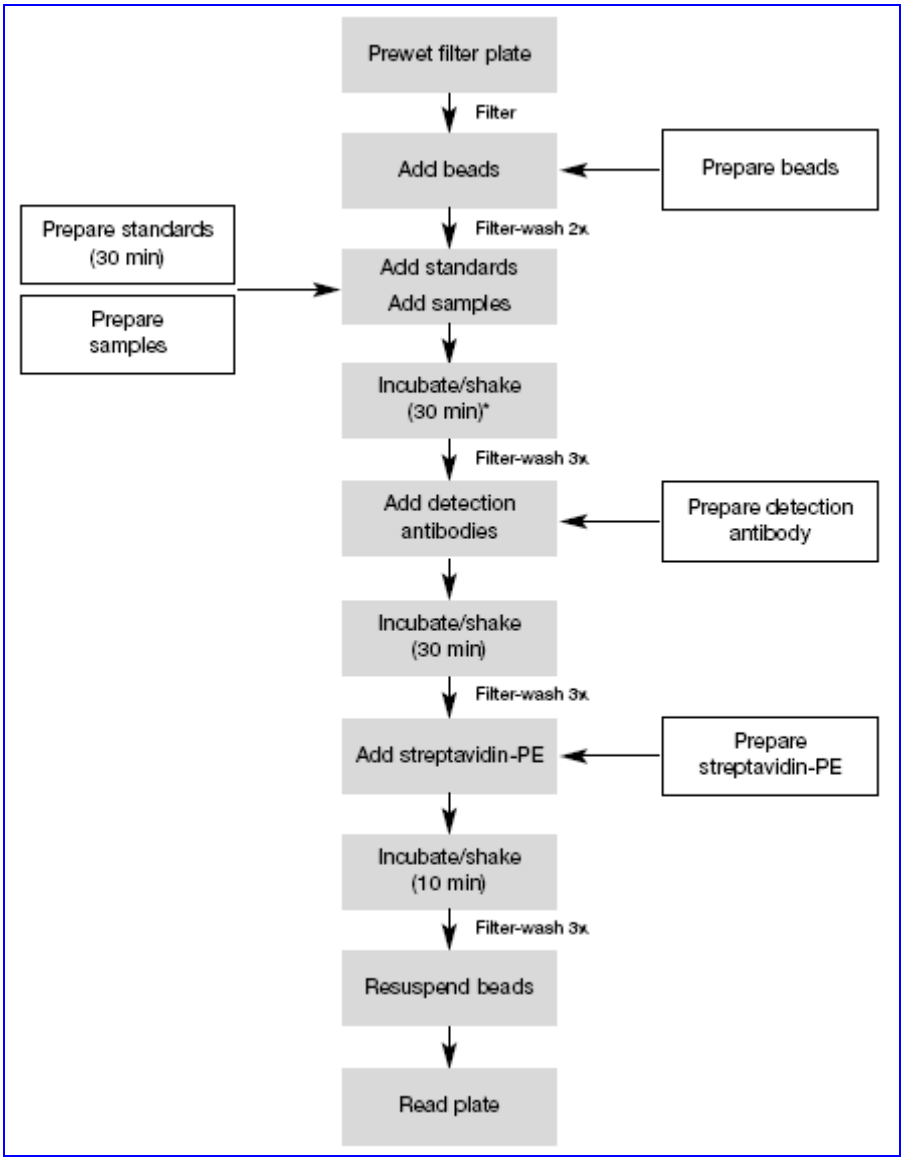


Fig. 39: *Determination of IL-6*

Washing steps were performed in 1.2 µm filter membrane 96-well microtiter plates (Millipore Corp., Billerica, MA) with a multi-screen vacuum manifold for 96-well plates (Millipore).
Incubations were done on a plate shaker Mini OrbitalShaker (Bellco Glass, In) at room temperature. The plates were read using the above mentioned Luminex100 System (Luminex Corp., Austin, TX).

C) A commercial ELISA kit (Monkey IL-1β – Cat # CMK039, Cell Sciences, Canton, MA) was used with its own reagents, buffers and standards, according to the leaflet upon an optimization phase. In particular, the standard curve was run in a pool of monkey plasma (Na-Citrate) instead of using the buffer provided by the vendor.

Data analysis:

A-B) Data were generated and analyzed using the Luminex dedicated software: these data were arranged in an Excel spreadsheet and subsequently visualized in GraphPad.

C) Data were generated using the Spectrophotometer dedicated software: these data were

analyzed and arranged in an Excel spreadsheet and subsequently visualized in GraphPad.

Statistical analysis was performed on GraphPad: a two-way ANOVA test with Bonferroni post-test (when necessary) was applied to evaluate both dose-effect along with time and time-effect within each experimental group. Due to the variability of the cytokine basal levels among animals, data were normalized versus the predose values.

4.2.3.1.10.3 Functional test: T-cell Proliferation assay and T_{reg} cells number evaluation

Functional tests were set to evaluate the antiproliferative and immunomodulatory effect of *Anti-CD25 mAb*.

CD25 is present at low levels in resting human T cells (with the exception of T regulatory cells) but is significantly up-regulated on activated T cells, enabling them to receive a high-affinity IL-2 signal [68].

Therefore, it is believed that the blockade of CD25 will result in selective functional inhibition of activated T cells [69]. Although it has been demonstrated that Daclizumab (or the original murine anti-Tac mAb) inhibits early IL-2R signal transduction events and blocks T cell activation and expansion in vitro [70], a comprehensive characterization of its *in vivo* effects is still lacking.

T-cell proliferation assay and *FoxP3 staining* were performed at the following time point: pre-dose, 24h after the last administration and at 3 recovery time-points (8, 18, 25 weeks after the last treatment).

Blood samples (3 ml/monkey) were collected in tubes with Na Heparin for PBMCs separation.

PBMCs from monkey were obtained by Ficoll gradient separation and $5x10^6$ cells were stained with CSFE 5 µM and $2x10^6$ unstained. Then cells were plated in a 96 well plate ($0.2x10^6$ cells/well) in complete medium (RPMI 10%FBS, 1%PS, 1%L-Gln, 1% NEN, 1‰ Hepes, 50 µM β- mercaptoethanol) in presence and absence of stimuli as IL2, PHA and CD3 and left at 37°C in a cell incubator. After 72h cell proliferation was analyzed by FACS analysis.

In parallel, PBMCs have been harvested immediately after blood collection for *FoxP3 staining*, in order to evaluate if the administration of *Anti-CD25 mAb* could affect T regulatory cells percentage.

Data from literature showed that in MS patients FoxP3 levels were diminished, indicating that an impaired immunoregulation by T regulatory cells could contribute to MS exacerbation [71].

4.2.3.1.10.4 Antibody determination

Antibody determination (i.e. the presence of host-anti-drug antibodies) was done using residual serum samples obtained pre-study, at the end of treatment and during the recovery period. Collected blood samples were allowed to clot for 50-60 minutes at room temperature.

The clot was spun down by centrifugation at 3000 rpm for 15 minutes and the supernatant was collected and divided into two aliquots, which were stored frozen at –80°C until analysis. Samples were analyzed using the validated ELISA method.

4.2.4 Statistical methods and data analysis

All raw data were recorded on appropriate forms bound in registers numbered, stored and processed by a computer system. All units of measure of the input data were selected so that the third decimal place would not be determinant. The computer rounds off figures at the second decimal place (except for the S.D.).

By Internal definition, “day 0” is the day immediately preceding the start of treatment (day 1).

The days of the experiment, both for pre-trial and experimental phases, are numbered according to this definition. In order to gather together the observations carried out on different calendar dates a “nominal day”, associated to each single observation, has been introduced.

This day, coincides with the actual treatment day (defined as the difference between the date of the event and the date relative to “day 0”, previously defined) only for body weight and food consumption measurements.

The “day”, is the actual day. The “week”, is calculated automatically on the basis of the “nominal day”, according to the following formula:

week = integer part [(nom. day + 6)/7] if nom. day greater or equal to 0
week = integer part [(nom. day - 6)/7] if nom. day less than 0

Recordings were expressed both in absolute figures and as mean and standard deviation (M. ± S.D.). All the data were compared according to following decision tree.

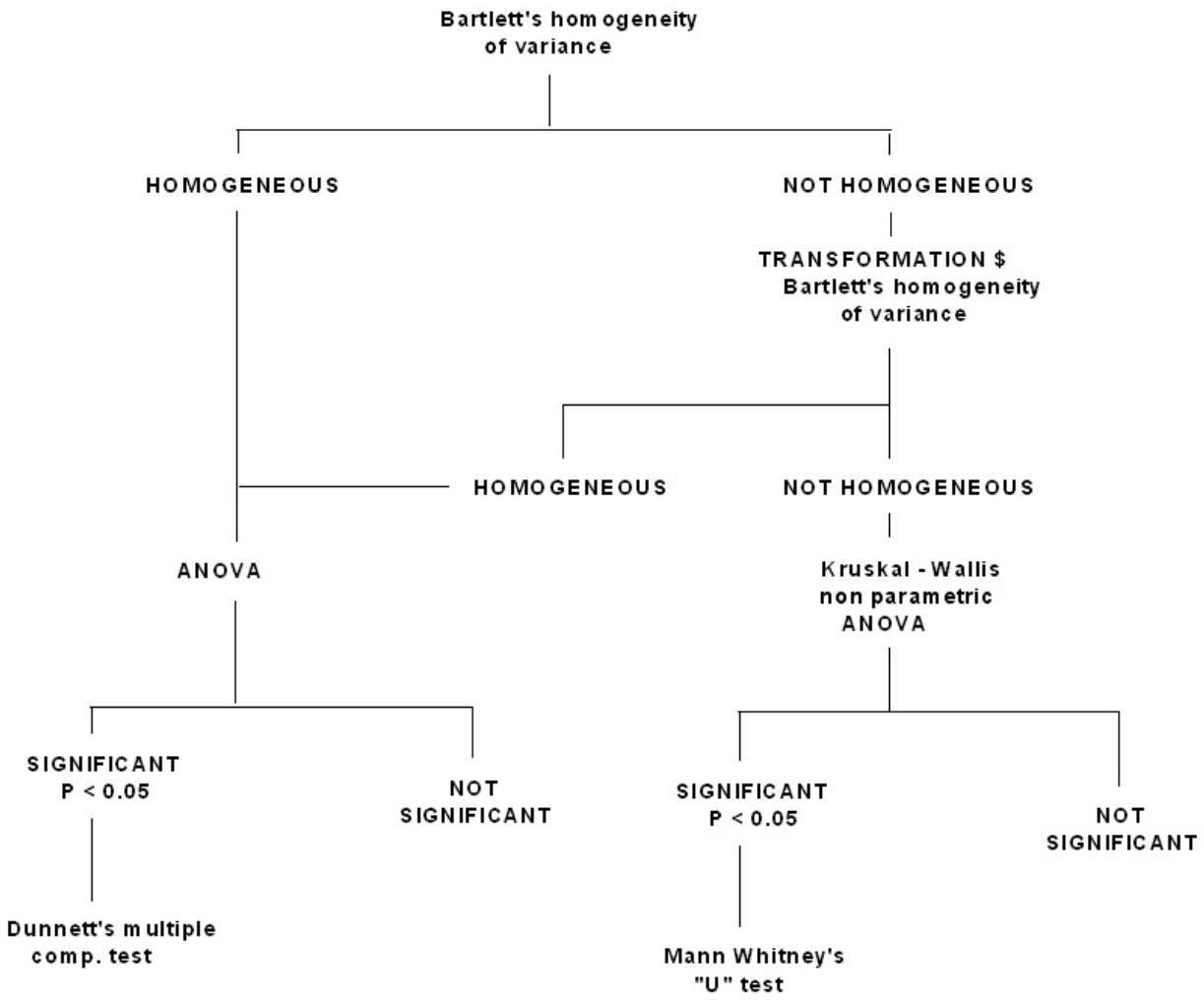


Fig. 40: *Statistical methods*

If one transformation was successfully applied, ANOVA and Dunnett's test (when necessary) were applied on transformed data.

Levels of significance were indicated by asterisks:

* P <0.05 (all tests)
** P <0.01 (all tests)
*** P <0.001 (all tests except Dunnett's test)

Statistical analysis was performed using Graphpad Prism 4.3. The analysis applied was a 2-way ANOVA with repeated measurements (matching by subject), considering the 2 factors Time and Dose, followed by Bonferroni as post hoc test.

4.2.5 Regulatory Compliance

The study design was in accordance with International Conference on Harmonization (ICH) Guidelines.

Procedures followed during the study were those documented in the test facility Standard Operating Procedures collection.

The Institute is fully authorized by Competent Veterinary Health Authorities.

4.2.6 Ethics

Protection of animals used in the experiment is in accordance with Directive 86/609/EEC, enforced by the Italian D. L. No. 116 of January 27, 1992.

Physical facilities and equipment for accommodation and care of animals are in accordance with the provisions of EEC Council Directive 86/609.

5. RESULTS

5.1 CLINICAL OBSERVATIONS

5.1.1 Mortality

No animals died during the study.

5.1.2 Clinical signs

No clinical signs (general and local) or behavioural changes were observed in any monkey.

5.1.3 Body Weight, Food Consumption, Rectal temperature

Statistical analysis of mean data did not reveal significant changes from the control group at any of the doses tested.

Treatment period - Males (monkeys)

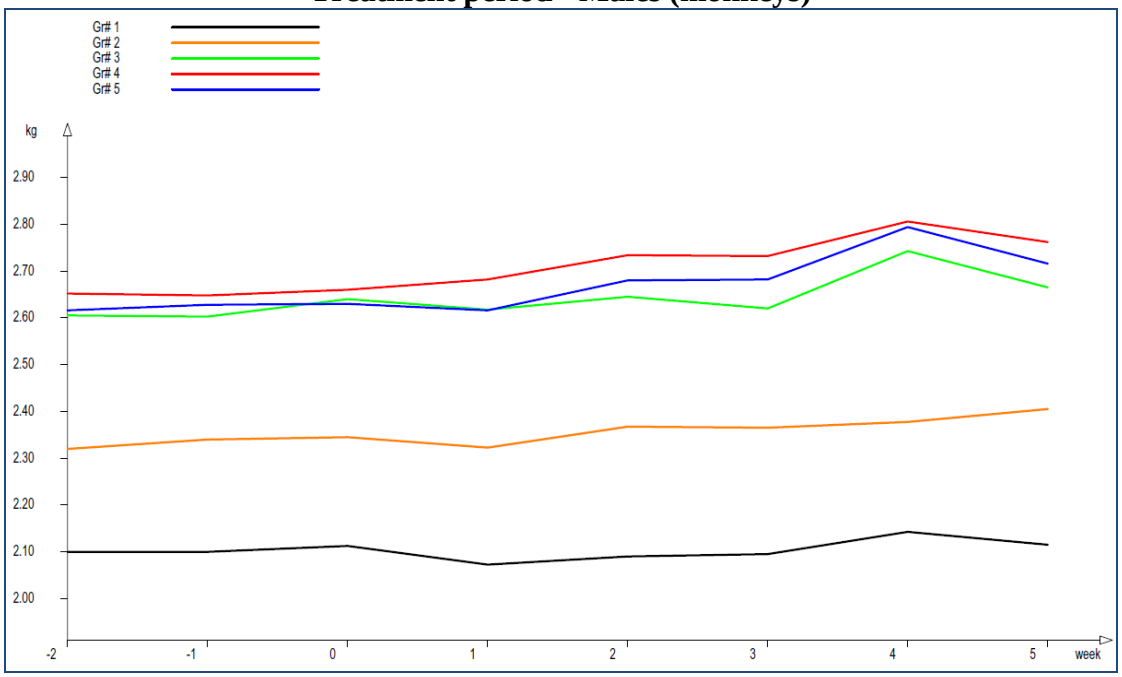


Fig. 41: *Body weight – Treatment period – All the animals: Males (monkeys)*
Gr1:0mg/kg IV;Gr2:5 mg/kg IV;Gr3: 25mg/kg IV;Gr4:125 mg/kg IV;Gr5: 75mg/kg SC

Treatment period - Females (monkeys)

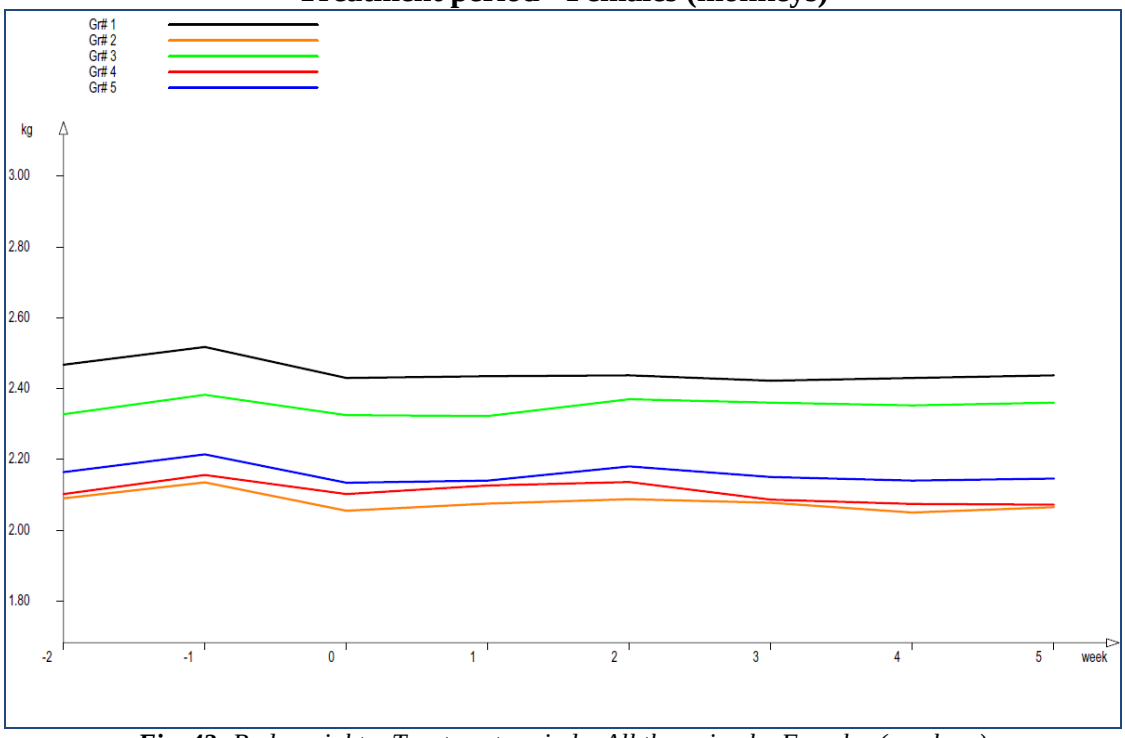


Fig. 42: *Body weight – Treatment period – All the animals: Females (monkeys)*
Gr1:0mg/kg IV; Gr2:5 mg/kg IV;Gr3: 25mg/kg IV;Gr4:125 mg/kg IV;Gr5: 75mg/kg SC

No adverse effects were seen on mean food consumption of animals treated with the test item at any dose.
No adverse effects were seen on body temperature. The differences highlighted by statistical analysis are without toxicological relevance.

5.1.4 Ophthalmological Examination

No eye changes were detected in any animal at any time.

5.1.5 Electrocardiography and heart rate

The subcutaneous and intravenous administration of *Anti-CD25 mAb* at all doses tested did not induce modifications in heart rate or in the electrocardiographic parameters. The morphological pattern of ECG complexes recorded at the scheduled times were comparable to those recorded pre-trial (basal) and no disturbances of cardiac rhythm were found at any dose.

5.2 LABORATORY INVESTIGATIOS

5.2.1 Hematology

No treatment-related hematological changes were seen in any animal treated at the various dosages by either intravenous or subcutaneous route at the end of the treatment or at any test point of the recovery period (***Tables 3and 4***).

A slight statistically significant decrease in fibrinogen was noted in males treated at 5 mg/kg/week pre-trial and at the end of the treatment period. However, it was considered incidental, being dose-unrelated.

Table 3: Hematologic parameters - Males (monkeys)
(Mean, S.D., n)

Dose (mg/kg/week)	Week	Gr# 1 0	Gr# 2 5	Gr# 3 25	Gr# 4 125	Gr# 5 75	ANOVA
Erythrocytes .....(x10E6/mcL)	-2	5.87 0.568 (4)	5.58 0.558 (4)	5.98 0.121 (4)	5.62 0.507 (5)	5.99 0.333 (5)	P
	6	5.02 0.555 (4)	4.70 0.464 (4)	5.01 0.347 (4)	4.95 0.356 (5)	5.12 0.305 (5)	P
	8	5.04 (1)	5.74 (1)	4.91 (1)	5.11 0.120 (2)	6.12 0.092 (2)	N
	13	5.34 (1)	6.02 (1)	5.55 (1)	5.54 0.064 (2)	6.49 0.198 (2)	N
	32	4.68 (1)	5.94 (1)	5.47 (1)	5.29 0.057 (2)	5.97 0.212 (2)	N
Hemoglobin .....(g/dL)	-2	14.00 0.638 (4)	13.35 0.465 (4)	14.72 0.550 (4)	13.60 0.704 (5)	13.94 0.868 (5)	P
	6	11.90 0.678 (4)	11.60 0.577 (4)	12.38 0.568 (4)	12.06 0.288 (5)	12.14 0.385 (5)	P
	8	12.70 (1)	12.40 (1)	12.80 (1)	12.50 0.424 (2)	13.90 0.000 (2)	N
	13	13.50 (1)	13.20 (1)	14.30 (1)	13.60 0.707 (2)	14.90 0.424 (2)	N
	32	12.20 (1)	13.00 (1)	14.00 (1)	13.25 0.212 (2)	13.90 0.566 (2)	N
Hematocrit .....(%)	-2	44.35 2.403 (4)	41.60 2.211 (4)	46.05 1.318 (4)	42.76 2.180 (5)	44.14 2.924 (5)	P
	6	37.85 2.024 (4)	35.97 1.305 (4)	38.48 1.852 (4)	37.64 1.184 (5)	37.46 1.282 (5)	P
	8	41.10 (1)	39.10 (1)	39.30 (1)	38.85 2.475 (2)	44.50 0.141 (2)	N
	13	43.10 (1)	40.40 (1)	44.30 (1)	42.05 2.333 (2)	47.15 2.475 (2)	N
	32	38.90 (1)	40.40 (1)	43.90 (1)	41.35 0.919 (2)	44.10 2.263 (2)	N

(Males - continue) Dose (mg/kg/week)		Gr# 1 0	Gr# 2 5	Gr# 3 25	Gr# 4 125	Gr# 5 75	ANOVA
	Week						
							P
Leukocytes .....(x10E3/mcL)	-2	14.20	11.21	13.18	12.47	10.02	
		2.846	3.038	5.162	3.404	3.003	
		(4)	(4)	(4)	(5)	(5)	
							N
	6	12.78	10.39	11.06	11.73	9.15	
		1.928	2.191	4.424	1.819	0.474	
		(4)	(4)	(4)	(5)	(5)	
							N
	8	8.31	9.21	7.01	11.75	9.63	
					0.778	1.930	
		(1)	(1)	(1)	(2)	(2)	
							N
	13	16.20	11.00	10.60	11.35	8.48	
					1.202	0.106	
		(1)	(1)	(1)	(2)	(2)	
							N
	32	15.80	10.60	15.10	14.45	8.88	
					1.485	0.884	
		(1)	(1)	(1)	(2)	(2)	
							P
Neutrophils .....(%)	-2	49.88	40.17	48.43	41.24	46.24	
		7.229	14.748	17.643	15.299	13.855	
		(4)	(4)	(4)	(5)	(5)	
							P
	6	47.98	38.65	50.65	41.53	50.86	
		10.364	13.126	9.956	23.409	13.906	
		(4)	(4)	(4)	(5)	(5)	
							N
	8	35.60	26.70	49.00	52.65	37.80	
					22.132	17.961	
		(1)	(1)	(1)	(2)	(2)	
							N
	13	27.80	28.90	32.20	42.35	31.05	
					4.172	10.536	
		(1)	(1)	(1)	(2)	(2)	
							N
	32	21.70	20.50	47.00	38.75	27.05	
					7.990	13.506	
		(1)	(1)	(1)	(2)	(2)	
							P
Lymphocytes .....(%)	-2	46.38	56.25	47.63	54.50	48.38	
		7.433	16.155	18.578	15.188	13.504	
		(4)	(4)	(4)	(5)	(5)	
							P
	6	47.70	55.92	44.62	52.42	44.84	
		9.487	12.515	11.383	21.875	13.175	
		(4)	(4)	(4)	(5)	(5)	
							N
	8	61.20	67.90	46.50	41.10	55.40	
					18.102	15.698	
		(1)	(1)	(1)	(2)	(2)	
							N
	13	68.10	67.60	65.50	54.05	62.90	
					0.071	10.041	
		(1)	(1)	(1)	(2)	(2)	
							N
	32	72.60	74.10	47.70	55.15	70.00	
					6.718	16.688	
		(1)	(1)	(1)	(2)	(2)	
							P
Monocytes .....(%)	-2	3.18	3.12	2.30	3.33	4.34	
		1.520	1.900	0.964	1.199	1.329	
		(4)	(4)	(4)	(5)	(5)	
							N
	6	3.28	4.53	3.41	4.68	3.21	
		0.670	2.461	1.133	2.831	0.388	
		(4)	(4)	(4)	(5)	(5)	

(Males - continue) Dose (mg/kg/week)		Gr# 1 0	Gr# 2 5	Gr# 3 25	Gr# 4 125	Gr# 5 75	ANOVA
	Week						
Monocytes .....(%)	8	2.66	4.70	2.45	5.51	6.08	N
					3.210	1.739	
		(1)	(1)	(1)	(2)	(2)	
	13	3.47	2.99	0.38	3.22	5.53	N
					4.356	0.445	
		(1)	(1)	(1)	(2)	(2)	
	32	4.29	4.84	2.62	4.92	2.39	N
					0.905	3.272	
		(1)	(1)	(1)	(2)	(2)	
Eosinophils .....(%)	-2	0.50	0.34	1.57	0.64	0.82	P
		0.253	0.159	1.285	0.539	0.628	
		(4)	(4)	(4)	(5)	(5)	
Eosinophils .....(%)	6	0.87	0.55	1.08	0.98	0.98	P
		0.647	0.378	0.375	0.904	0.727	
		(4)	(4)	(4)	(5)	(5)	
	8	0.31	0.38	1.87	0.23	0.43	N
					0.267	0.109	
		(1)	(1)	(1)	(2)	(2)	
	13	0.64	0.43	1.93	0.31	0.45	N
					0.202	0.081	
		(1)	(1)	(1)	(2)	(2)	
	32	1.32	0.28	2.65	0.92	0.49	N
					0.486	0.178	
		(1)	(1)	(1)	(2)	(2)	
Basophils .....(%)	-2	0.10	0.14	0.07	0.27	0.19	N
		0.128	0.128	0.035	0.179	0.132	
		(4)	(4)	(4)	(5)	(5)	
	6	0.20	0.32	0.20	0.40	0.11	N
		0.083	0.350	0.275	0.588	0.059	
		(4)	(4)	(4)	(5)	(5)	
	8	0.23	0.35	0.12	0.51	0.33	N
					0.543	0.390	
		(1)	(1)	(1)	(2)	(2)	
	13	0.06	0.06	0.00	0.10	0.07	N
					0.134	0.020	
		(1)	(1)	(1)	(2)	(2)	
	32	0.12	0.32	0.06	0.31	0.06	N
					0.062	0.078	
		(1)	(1)	(1)	(2)	(2)	
Neutrophils .....(x10E3/mcL)	-2	7.14	4.58	7.02	5.16	4.86	P
		2.039	2.302	5.394	2.651	2.640	
		(4)	(4)	(4)	(5)	(5)	
	6	6.20	4.17	5.87	5.00	4.64	P
		1.996	1.956	3.475	3.173	1.258	
		(4)	(4)	(4)	(5)	(5)	
	8	2.96	2.46	3.44	6.27	3.80	N
					2.998	2.447	
		(1)	(1)	(1)	(2)	(2)	
	13	4.49	3.17	3.42	4.79	2.63	N
					0.049	0.863	
		(1)	(1)	(1)	(2)	(2)	

(Males - continue)		Gr# 1	Gr# 2	Gr# 3	Gr# 4	Gr# 5	ANOVA
Dose (mg/kg/week)		0	5	25	125	75	
	Week						
							N
Neutrophils .....(x10E3/mcL)	32	3.44	2.17	7.11	5.54	2.35	
					0.601	0.969	
		(1)	(1)	(1)	(2)	(2)	
							P
Lymphocytes .....(x10E3/mcL)	-2	6.53	6.23	5.58	6.77	4.61	
		1.460	2.382	0.662	2.396	0.840	
		(4)	(4)	(4)	(5)	(5)	
							P
	6	6.04	5.66	4.65	6.03	4.12	
		1.250	0.952	1.192	2.475	1.301	
		(4)	(4)	(4)	(5)	(5)	
							N
	8	5.09	6.25	3.26	4.77	5.18	
					1.831	0.460	
		(1)	(1)	(1)	(2)	(2)	
							N
	13	11.00	7.42	6.97	6.13	5.34	
					0.672	0.919	
		(1)	(1)	(1)	(2)	(2)	
							N
	32	11.50	7.86	7.21	8.01	6.29	
					1.768	2.100	
		(1)	(1)	(1)	(2)	(2)	
							P
Monocytes .....(x10E3/mcL)	-2	0.44	0.35	0.32	0.45	0.45	
		0.212	0.229	0.248	0.229	0.221	
		(4)	(4)	(4)	(5)	(5)	
							N
	6	0.42	0.48	0.41	0.53	0.29	
		0.106	0.341	0.292	0.332	0.030	
		(4)	(4)	(4)	(5)	(5)	
							N
	8	0.22	0.43	0.17	0.64	0.57	
					0.337	0.052	
		(1)	(1)	(1)	(2)	(2)	
							N
	13	0.56	0.33	0.04	0.39	0.47	
					0.535	0.044	
		(1)	(1)	(1)	(2)	(2)	
							N
	32	0.68	0.51	0.40	0.72	0.20	
					0.202	0.270	
		(1)	(1)	(1)	(2)	(2)	
							P
Eosinophils .....(x10E3/mcL)	-2	0.07	0.04	0.22	0.07	0.08	
		0.048	0.021	0.196	0.032	0.075	
		(4)	(4)	(4)	(5)	(5)	
							P
	6	0.12	0.06	0.12	0.12	0.09	
		0.096	0.049	0.048	0.103	0.067	
		(4)	(4)	(4)	(5)	(5)	
							N
	8	0.03	0.04	0.13	0.03	0.04	
					0.030	0.002	
		(1)	(1)	(1)	(2)	(2)	
							N
	13	0.10	0.05	0.21	0.03	0.04	
					0.019	0.008	
		(1)	(1)	(1)	(2)	(2)	
							N
	32	0.21	0.03	0.40	0.14	0.04	
					0.083	0.020	
		(1)	(1)	(1)	(2)	(2)	
							P
Basophils .....(x10E3/mcL)	-2	0.01	0.02	0.01	0.04	0.02	
		0.019	0.015	0.005	0.027	0.019	
		(4)	(4)	(4)	(5)	(5)	

(Males - continue) Dose (mg/kg/week)		Gr# 1 0	Gr# 2 5	Gr# 3 25	Gr# 4 125	Gr# 5 75	ANOVA
	Week						
Basophils .....(x10E3/mcL)	6	0.03	0.03	0.03	0.05	0.01	N
		0.009	0.044	0.048	0.071	0.006	
		(4)	(4)	(4)	(5)	(5)	
	8	0.02	0.03	0.01	0.06	0.03	N
					0.060	0.031	
		(1)	(1)	(1)	(2)	(2)	
	13	0.01	0.01	0.00	0.01	0.01	N
					0.016	0.001	
		(1)	(1)	(1)	(2)	(2)	
	32	0.02	0.03	0.01	0.04	0.00	N
					0.005	0.006	
		(1)	(1)	(1)	(2)	(2)	
Mean Corpuscular Volume .....(fL)	-2	75.90	74.92	77.00	76.24	73.62	P
		4.745	6.912	2.340	4.279	2.226	
		(4)	(4)	(4)	(5)	(5)	
	6	75.95	77.03	77.00	76.20	73.30	P
		5.429	7.198	2.736	3.750	2.564	
		(4)	(4)	(4)	(5)	(5)	
Mean Corpuscular Volume .....(fL)	8	81.40	68.10	80.00	76.05	72.60	N
					3.041	0.849	
		(1)	(1)	(1)	(2)	(2)	
	13	80.70	67.20	79.80	75.80	72.55	N
					3.394	1.626	
		(1)	(1)	(1)	(2)	(2)	
	32	83.00	68.00	80.30	78.25	73.85	N
					2.475	1.202	
		(1)	(1)	(1)	(2)	(2)	
Mean Corpuscular HGB Conc. .....(g/dL)	-2	31.57	32.12	32.00	31.84	31.62	P
		0.591	0.655	0.510	0.619	0.698	
		(4)	(4)	(4)	(5)	(5)	
	6	31.40	32.23	32.25	32.00	32.38	N
		0.082	0.427	0.705	0.778	0.370	
		(4)	(4)	(4)	(5)	(5)	
	8	30.90	31.80	32.50	32.15	31.30	N
					1.061	0.000	
		(1)	(1)	(1)	(2)	(2)	
	13	31.40	32.60	32.30	32.35	31.60	N
					0.071	0.849	
		(1)	(1)	(1)	(2)	(2)	
	32	31.30	32.10	31.90	32.10	31.45	N
					0.141	0.354	
		(1)	(1)	(1)	(2)	(2)	
Mean Corpuscular HGB .....(pg)	-2	23.98	24.05	24.65	24.32	23.28	P
		1.452	2.179	1.008	1.801	1.006	
		(4)	(4)	(4)	(5)	(5)	
	6	23.85	24.85	24.85	24.42	23.72	P
		1.654	2.339	1.363	1.647	1.055	
		(4)	(4)	(4)	(5)	(5)	
	8	25.20	21.70	26.00	24.45	22.70	N
					0.212	0.283	
		(1)	(1)	(1)	(2)	(2)	

(Males - continue) Dose (mg/kg/week)		Gr# 1 0	Gr# 2 5	Gr# 3 25	Gr# 4 125	Gr# 5 75	ANOVA	
	Week							
Mean Corpuscular HGB .....(pg)	13	25.40	21.90	25.80	24.55	22.95	N	
					1.061	0.071		
		(1)	(1)	(1)	(2)	(2)		
	32	26.00	21.90	25.60	25.15	23.25	N	
					0.636	0.071		
		(1)	(1)	(1)	(2)	(2)		
Platelets .....(x10E3/mcL)	-2	264.75	323.00	353.50	312.20	320.80	P	
		30.391	54.522	79.910	64.794	98.594		
		(4)	(4)	(4)	(5)	(5)		
	6	283.25	306.00	409.25	342.00	341.40	P	
		43.146	51.088	67.638	40.927	103.057		
		(4)	(4)	(4)	(5)	(5)		
	8	275.00	325.00	420.00	355.00	324.50	N	
					77.782	71.418		
		(1)	(1)	(1)	(2)	(2)		
	13	342.00	381.00	446.00	370.50	369.00	N	
					106.773	39.598		
		(1)	(1)	(1)	(2)	(2)		
	32	418.00	389.00	444.00	383.50	339.50	N	
					120.915	75.660		
		(1)	(1)	(1)	(2)	(2)		
Prothrombin Time .....(sec)	-2	9.89	9.75	9.11	9.25	9.62	N	
		1.567	0.733	0.295	0.391	2.731		
		(4)	(4)	(4)	(5)	(5)		
	6	10.18	10.60	10.18	10.34	10.38	P	
		0.859	0.572	0.544	0.391	0.415		
		(4)	(4)	(4)	(5)	(5)		
	8	10.90	13.50	13.70	13.25	12.65	N	
					1.909	0.919		
		(1)	(1)	(1)	(2)	(2)		
	13	9.90	10.20	9.60	9.98	10.15	N	
					0.318	0.071		
		(1)	(1)	(1)	(2)	(2)		
	32	8.85	9.30	9.30	9.45	9.68	N	
					0.000	0.106		
		(1)	(1)	(1)	(2)	(2)		
Fibrinogen .....(mg/dL)	-2	272.25	195.50 **	249.50	252.40	244.20	P	*
		31.742	9.469	26.489	36.060	34.967		
		(4)	(4)	(4)	(5)	(5)		
	1	271.50	234.00	296.00	284.25	288.60	P	
		58.666	26.981	63.003	49.284	45.357		
		(4)	(4)	(4)	(4)	(5)		
	3	254.75	226.25	251.25	271.60	247.40	P	
		11.587	17.858	13.623	22.367	33.687		
		(4)	(4)	(4)	(5)	(5)		
	3	267.50	247.50	280.25	290.60	264.80	P	
		9.000	27.887	24.432	20.157	45.263		
		(4)	(4)	(4)	(5)	(5)		
	5	261.25	240.00	291.00	298.40	240.20	P	
		25.734	21.710	36.092	62.035	36.711		
		(4)	(4)	(4)	(5)	(5)		

(Males - continue)		Gr# 1	Gr# 2	Gr# 3	Gr# 4	Gr# 5	ANOVA
Dose (mg/kg/week)		0	5	25	125	75	
	Week						
Fibrinogen .....(mg/dL)	5	295.50	255.50	311.25	355.00	308.80	P
		30.512	35.940	33.896	60.092	47.819	
		(4)	(4)	(4)	(5)	(5)	
	6	306.25	202.25 **	253.00	276.20	273.20	P **
		28.418	12.868	22.465	33.064	41.656	
		(4)	(4)	(4)	(5)	(5)	
	8	241.00	238.00	251.00	275.00	268.50	N
					52.326	38.891	
		(1)	(1)	(1)	(2)	(2)	
	13	207.00	207.00	212.00	223.00	241.00	N
					0.000	18.385	
		(1)	(1)	(1)	(2)	(2)	
	32	245.00	224.00	254.00	262.00	276.00	N
					49.497	7.071	
		(1)	(1)	(1)	(2)	(2)	
Activated Partial Thromboplastin Time .....(sec)	-2	17.77	19.38	18.27	18.98	18.84	P
		0.914	1.788	1.873	1.137	1.345	
		(4)	(4)	(4)	(5)	(5)	
	6	19.08	21.62	19.80	21.10	19.24	P
		0.991	0.613	1.467	1.202	2.638	
		(4)	(4)	(4)	(5)	(5)	
	8	18.00	23.20	20.40	24.55	20.30	N
					3.323	2.828	
		(1)	(1)	(1)	(2)	(2)	
Activated Partial Thromboplastin Time .....(sec)	13	17.50	16.60	16.00	17.65	15.50	N
					1.202	1.273	
		(1)	(1)	(1)	(2)	(2)	
	32	16.00	15.90	15.10	18.00	15.90	N
					0.849	0.707	
		(1)	(1)	(1)	(2)	(2)	

ANOVA: "P" = Parametric; "N" = Non Parametric; "-" = Not processed
Significance Level: * p < 0.05; ** p < 0.01; *** p < 0.001

Table 4: Hematologic parameters - Famales (monkeys)
(Mean, S.D., n)

		Gr# 1	Gr# 2	Gr# 3	Gr# 4	Gr# 5	ANOVA
Dose (mg/kg/week)		0	5	25	125	75	
	Week						
Erythrocytes .....(x10E6/mcL)	-2	5.60	5.60	5.67	5.59	5.61	P
		0.419	0.292	0.505	0.464	0.303	
		(4)	(4)	(4)	(5)	(5)	
	6	4.65	4.66	4.64	4.76	4.96	P
		0.382	0.260	0.436	0.556	0.316	
		(4)	(4)	(4)	(5)	(5)	
	8	5.40	5.40	5.45	5.75	5.36	N
					0.438	0.064	
		(1)	(1)	(1)	(2)	(2)	
	13	5.53	5.68	5.59	6.12	5.66	N
					0.177	0.163	
		(1)	(1)	(1)	(2)	(2)	

(Females - continue)		Gr# 1	Gr# 2	Gr# 3	Gr# 4	Gr# 5	ANOVA
Dose (mg/kg/week)		0	5	25	125	75	
	Week						
							N
	32	5.50	5.73	5.56	5.95	5.64	
					0.523	0.495	
		(1)	(1)	(1)	(2)	(2)	
							P
Hemoglobin .....(g/dL)	-2	13.28	13.50	13.65	13.34	13.24	
		1.239	0.812	1.201	0.805	0.513	
		(4)	(4)	(4)	(5)	(5)	
							P
	6	11.10	11.28	11.30	11.32	11.62	
		0.876	0.340	0.757	0.753	0.653	
		(4)	(4)	(4)	(5)	(5)	
							N
	8	13.60	12.70	13.30	13.25	12.45	
					1.061	0.354	
		(1)	(1)	(1)	(2)	(2)	
							N
	13	14.10	13.40	13.60	13.80	12.95	
					0.424	0.354	
		(1)	(1)	(1)	(2)	(2)	
							N
	32	13.90	13.40	13.60	13.90	13.25	
					1.131	0.495	
		(1)	(1)	(1)	(2)	(2)	
							P
Hematocrit .....(%)	-2	41.92	42.30	42.85	41.64	41.82	
		4.155	2.667	4.007	2.460	1.232	
		(4)	(4)	(4)	(5)	(5)	
							P
	6	35.28	35.58	35.60	36.04	37.10	
		3.099	1.072	3.058	2.801	1.789	
		(4)	(4)	(4)	(5)	(5)	
							N
	8	42.30	39.70	40.90	41.15	39.25	
					2.475	1.202	
		(1)	(1)	(1)	(2)	(2)	
							N
	13	42.80	41.70	41.60	44.00	41.15	
					0.566	0.354	
		(1)	(1)	(1)	(2)	(2)	
							N
	32	43.50	42.70	42.70	43.35	41.85	
					3.182	2.475	
		(1)	(1)	(1)	(2)	(2)	
							P
Leukocytes .....(x10E3/mcL)	-2	12.69	12.15	13.08	13.83	16.32	
		2.952	2.344	4.025	5.053	5.777	
		(4)	(4)	(4)	(5)	(5)	
							P
	6	14.41	15.39	11.72	13.84	11.88	
		6.895	4.312	1.884	7.589	2.480	
		(4)	(4)	(4)	(5)	(5)	
							N
	8	12.50	10.80	9.57	10.11	13.75	
					1.683	3.889	
		(1)	(1)	(1)	(2)	(2)	
							N
Leukocytes .....(x10E3/mcL)	13	12.80	9.69	15.10	12.26	14.40	
					4.306	2.970	
		(1)	(1)	(1)	(2)	(2)	
							N
	32	17.60	11.80	9.45	13.70	16.30	
					0.424	1.556	
		(1)	(1)	(1)	(2)	(2)	
							P
Neutrophils .....(%)	-2	27.73	33.80	29.57	37.84	31.00	
		10.338	8.722	9.534	23.541	4.741	
		(4)	(4)	(4)	(5)	(5)	

(Females - continue) Dose (mg/kg/week)		Gr# 1 0	Gr# 2 5	Gr# 3 25	Gr# 4 125	Gr# 5 75	ANOVA
	Week						
Neutrophils .....(%)	6	44.10	56.65	38.20	50.22	35.00	P
		16.371	14.776	18.122	22.159	10.050	
		(4)	(4)	(4)	(5)	(5)	
	8	38.90	41.40	30.60	34.50	33.25	N
					28.284	3.182	
		(1)	(1)	(1)	(2)	(2)	
	13	31.80	19.80	27.10	39.05	20.50	N
					19.163	2.687	
		(1)	(1)	(1)	(2)	(2)	
	32	51.30	28.80	37.90	45.95	24.70	N
					24.678	8.061	
		(1)	(1)	(1)	(2)	(2)	
Lymphocytes .....(%)	-2	65.10	56.98	64.25	56.18	63.92	N
		10.120	5.324	9.750	25.094	3.770	
		(4)	(4)	(4)	(5)	(5)	
	6	47.98	38.33	55.75	44.62	58.44	P
		12.167	13.738	17.803	22.012	7.966	
		(4)	(4)	(4)	(5)	(5)	
	8	55.20	52.30	60.90	55.85	60.40	N
					34.153	2.828	
		(1)	(1)	(1)	(2)	(2)	
	13	65.40	72.80	66.30	53.35	73.55	N
					20.153	0.495	
		(1)	(1)	(1)	(2)	(2)	
	32	43.90	67.10	61.80	48.40	70.70	N
					27.294	7.637	
		(1)	(1)	(1)	(2)	(2)	
Monocytes .....(%)	-2	4.73	6.66	4.66	4.21	3.68	P
		1.043	2.751	0.709	2.636	2.023	
		(4)	(4)	(4)	(5)	(5)	
	6	5.93	3.97	4.71	4.26	4.90	P
		3.458	2.642	1.777	1.123	2.253	
		(4)	(4)	(4)	(5)	(5)	
	8	4.75	5.04	7.03	7.84	4.74	N
					6.173	0.997	
		(1)	(1)	(1)	(2)	(2)	
	13	1.54	5.19	4.93	5.84	3.94	N
					2.376	1.450	
		(1)	(1)	(1)	(2)	(2)	
	32	3.70	3.32	0.08	3.53	3.15	N
					4.472	0.000	
		(1)	(1)	(1)	(2)	(2)	
Eosinophils .....(%)	-2	2.21	2.07	1.16	1.52	1.20	P
		3.173	1.950	0.869	1.157	0.496	
		(4)	(4)	(4)	(5)	(5)	
	6	1.58	0.70	1.12	0.76	1.32	P
		1.479	0.733	1.223	0.594	0.926	
		(4)	(4)	(4)	(5)	(5)	
	8	1.06	1.24	1.41	1.59	1.43	N
					0.354	1.299	
		(1)	(1)	(1)	(2)	(2)	

(Females - continue) Dose (mg/kg/week)		Gr# 1 0	Gr# 2 5	Gr# 3 25	Gr# 4 125	Gr# 5 75	ANOVA
	Week						
Eosinophils .....(%)	13	0.97	2.23	1.62	1.68	1.64	N
					1.319	1.386	
		(1)	(1)	(1)	(2)	(2)	
	32	1.01	0.74	0.21	2.03	1.25	N
					1.907	0.537	
		(1)	(1)	(1)	(2)	(2)	
Basophils .....(%)	-2	0.24	0.47	0.34	0.21	0.20	N
		0.157	0.470	0.316	0.200	0.165	
		(4)	(4)	(4)	(5)	(5)	
	6	0.42	0.37	0.22	0.16	0.36	N
		0.424	0.293	0.167	0.121	0.347	
		(4)	(4)	(4)	(5)	(5)	
Basophils .....(%)	8	0.14	0.07	0.10	0.28	0.14	N
					0.084	0.081	
		(1)	(1)	(1)	(2)	(2)	
	13	0.24	0.00	0.11	0.12	0.38	N
					0.027	0.299	
		(1)	(1)	(1)	(2)	(2)	
	32	0.07	0.08	0.00	0.03	0.16	N
					0.039	0.105	
		(1)	(1)	(1)	(2)	(2)	
Neutrophils .....(x10E3/mcL)	-2	3.59	4.17	3.91	5.48	4.88	P
		1.685	1.522	1.973	4.947	1.006	
		(4)	(4)	(4)	(5)	(5)	
	6	7.16	8.59	4.55	7.55	4.05	P
		5.798	3.088	2.697	6.854	0.986	
		(4)	(4)	(4)	(5)	(5)	
	8	4.87	4.48	2.93	3.25	4.51	N
					2.284	0.870	
		(1)	(1)	(1)	(2)	(2)	
Neutrophils .....(x10E3/mcL)	13	4.07	1.92	4.09	4.36	2.91	N
					0.679	0.219	
		(1)	(1)	(1)	(2)	(2)	
	32	9.05	3.39	3.58	6.34	3.96	N
					3.578	0.926	
		(1)	(1)	(1)	(2)	(2)	
Lymphocytes .....(x10E3/mcL)	-2	8.14	6.92	8.38	7.50	10.58	P
		1.770	1.467	2.874	3.198	4.425	
		(4)	(4)	(4)	(5)	(5)	
	6	6.31	6.05	6.50	5.57	7.02	P
		1.445	3.389	2.433	2.459	2.098	
		(4)	(4)	(4)	(5)	(5)	
	8	6.92	5.66	5.83	5.92	8.35	N
					4.377	2.758	
		(1)	(1)	(1)	(2)	(2)	
	13	8.37	7.05	10.00	6.95	10.59	N
					4.738	2.128	
		(1)	(1)	(1)	(2)	(2)	
	32	7.74	7.89	5.84	6.56	11.56	N
					3.521	2.319	
		(1)	(1)	(1)	(2)	(2)	

(Females - continue) Dose (mg/kg/week)		Gr# 1 0	Gr# 2 5	Gr# 3 25	Gr# 4 125	Gr# 5 75	ANOVA
	Week						
							P
Monocytes .....(x10E3/mcL)	-2	0.60	0.77	0.63	0.54	0.63	
		0.210	0.216	0.246	0.312	0.487	
		(4)	(4)	(4)	(5)	(5)	
							P
	6	0.68	0.57	0.53	0.55	0.59	
		0.157	0.382	0.115	0.207	0.319	
		(4)	(4)	(4)	(5)	(5)	
							N
	8	0.59	0.55	0.67	0.74	0.63	
					0.487	0.050	
		(1)	(1)	(1)	(2)	(2)	
							N
	13	0.20	0.50	0.74	0.66	0.59	
					0.041	0.325	
		(1)	(1)	(1)	(2)	(2)	
							N
	32	0.65	0.39	0.01	0.49	0.51	
					0.627	0.048	
		(1)	(1)	(1)	(2)	(2)	
							P
Eosinophils .....(x10E3/mcL)	-2	0.33	0.23	0.13	0.25	0.19	
		0.507	0.190	0.105	0.248	0.094	
		(4)	(4)	(4)	(5)	(5)	
							P
	6	0.21	0.11	0.12	0.13	0.17	
		0.229	0.129	0.130	0.157	0.130	
		(4)	(4)	(4)	(5)	(5)	
							N
	8	0.13	0.13	0.14	0.16	0.22	
					0.062	0.235	
		(1)	(1)	(1)	(2)	(2)	
							N
	13	0.12	0.22	0.24	0.23	0.26	
					0.233	0.247	
		(1)	(1)	(1)	(2)	(2)	
							N
	32	0.18	0.09	0.02	0.27	0.21	
					0.252	0.107	
		(1)	(1)	(1)	(2)	(2)	
							P
Basophils .....(x10E3/mcL)	-2	0.03	0.05	0.05	0.03	0.04	
		0.024	0.046	0.048	0.025	0.044	
		(4)	(4)	(4)	(5)	(5)	
							P
	6	0.05	0.06	0.03	0.02	0.04	
		0.030	0.055	0.021	0.021	0.043	
		(4)	(4)	(4)	(5)	(5)	
							N
	8	0.02	0.01	0.01	0.03	0.02	
					0.004	0.017	
		(1)	(1)	(1)	(2)	(2)	
							N
	13	0.03	0.00	0.02	0.02	0.06	
					0.008	0.054	
		(1)	(1)	(1)	(2)	(2)	
							N
	32	0.01	0.01	0.00	0.00	0.03	
					0.006	0.015	
		(1)	(1)	(1)	(2)	(2)	
							P
Mean Corpuscular Volume .....(fL)	-2	74.92	75.60	75.50	74.66	74.56	
		3.035	2.041	3.093	2.967	1.988	
		(4)	(4)	(4)	(5)	(5)	
							P
	6	75.92	76.50	76.78	75.96	74.92	
		2.939	2.146	2.024	3.250	2.799	
		(4)	(4)	(4)	(5)	(5)	

(Females - continue) Dose (mg/kg/week)		Gr# 1 0	Gr# 2 5	Gr# 3 25	Gr# 4 125	Gr# 5 75	ANOVA
	Week						
Mean Corpuscular Volume .....(fL)	8	78.30	73.50	75.00	71.60	73.40	N
					1.131	3.111	
		(1)	(1)	(1)	(2)	(2)	
	13	77.50	73.40	74.50	71.95	72.85	N
					1.202	2.758	
		(1)	(1)	(1)	(2)	(2)	
	32	79.20	74.50	76.70	72.90	74.25	N
					1.131	2.051	
		(1)	(1)	(1)	(2)	(2)	
Mean Corpuscular HGB Conc. .....(g/dL)	-2	31.63	31.93	31.90	32.08	31.60	P
		0.310	0.411	0.775	0.342	0.583	
		(4)	(4)	(4)	(5)	(5)	
	6	31.53	31.73	31.80	31.42	31.40	P
		0.723	0.287	0.698	0.841	0.612	
		(4)	(4)	(4)	(5)	(5)	
	8	32.10	32.10	32.50	32.20	31.75	N
					0.566	0.071	
		(1)	(1)	(1)	(2)	(2)	
	13	32.90	32.30	32.60	31.45	31.40	N
					0.636	0.566	
		(1)	(1)	(1)	(2)	(2)	
	32	32.00	31.50	31.90	32.15	31.65	N
					0.212	0.636	
		(1)	(1)	(1)	(2)	(2)	
Mean Corpuscular HGB .....(pg)	-2	23.68	24.12	24.05	23.94	23.56	P
		0.900	0.486	0.719	1.092	0.688	
		(4)	(4)	(4)	(5)	(5)	
	6	23.95	24.27	24.40	23.90	23.52	P
		1.258	0.660	0.753	1.622	0.918	
		(4)	(4)	(4)	(5)	(5)	
	8	25.10	23.50	24.40	23.05	23.30	N
					0.071	0.990	
		(1)	(1)	(1)	(2)	(2)	
	13	25.50	23.70	24.30	22.55	22.85	N
					0.071	1.344	
		(1)	(1)	(1)	(2)	(2)	
	32	25.30	23.50	24.50	23.45	23.50	N
					0.212	1.131	
		(1)	(1)	(1)	(2)	(2)	
Platelets .....(x10E3/mcL)	-2	325.00	301.25	339.75	400.80	283.00	P
		30.463	44.131	84.721	49.160	85.121	
		(4)	(4)	(4)	(5)	(5)	
	6	354.00	347.25	329.75	413.00	340.80	P
		110.686	44.925	80.769	24.970	65.473	
		(4)	(4)	(4)	(5)	(5)	
	8	279.00	233.00	395.00	369.00	280.00	N
					5.657	113.137	
		(1)	(1)	(1)	(2)	(2)	
	13	314.00	234.00	373.00	386.50	313.50	N
					12.021	137.886	
		(1)	(1)	(1)	(2)	(2)	

(Females - continue)		Gr# 1	Gr# 2	Gr# 3	Gr# 4	Gr# 5	ANOVA
Dose (mg/kg/week)		0	5	25	125	75	
	Week						
Platelets .....(x10E3/mcL)	32	317.00	302.00	452.00	422.00	362.50	N
					26.870	116.673	
		(1)	(1)	(1)	(2)	(2)	
Prothrombin Time .....(sec)	-2	9.95	9.97	10.38	10.14	10.22	P
		0.858	0.806	0.189	0.416	0.550	
		(4)	(4)	(4)	(5)	(5)	
	6	10.25	9.95	10.83	10.04	10.18	P
		0.332	0.603	0.512	0.230	0.502	
		(4)	(4)	(4)	(5)	(5)	
	8	10.50	10.50	13.60	10.07	10.65	N
					0.460	0.354	
		(1)	(1)	(1)	(2)	(2)	
	13	12.40	10.30	11.30	10.10	9.85	N
					0.424	0.636	
		(1)	(1)	(1)	(2)	(2)	
	32	9.00	10.50	10.60	9.38	10.03	N
					1.167	0.813	
		(1)	(1)	(1)	(2)	(2)	
Fibrinogen .....(mg/dL)	-2	219.00	232.75	227.25	219.60	242.20	P
		72.392	28.395	38.012	19.680	19.422	
		(4)	(4)	(4)	(5)	(5)	
	1	271.75	261.25	275.00	330.80	387.40	P
		20.549	39.204	49.173	89.438	102.810	
		(4)	(4)	(4)	(5)	(5)	
	3	275.25	252.75	226.25	226.40	252.60	P
		53.922	54.402	28.312	26.633	17.516	
		(4)	(4)	(4)	(5)	(5)	
	3	282.25	262.50	237.75	240.80	249.80	P
		62.591	44.918	18.786	16.664	12.716	
		(4)	(4)	(4)	(5)	(5)	
	5	283.50	334.75	222.00	234.00	246.80	P
		79.685	219.505	67.562	21.668	28.683	
		(4)	(4)	(4)	(5)	(5)	
	5	250.50	334.25	237.75	278.00	248.60	N
		82.075	219.922	71.955	49.188	15.258	
		(4)	(4)	(4)	(5)	(5)	
	6	250.75	263.50	222.50	233.20	225.00	P
		48.965	89.161	81.193	37.772	11.554	
		(4)	(4)	(4)	(5)	(5)	
	8	184.00	160.00	209.00	167.00	189.00	N
					15.556	1.414	
		(1)	(1)	(1)	(2)	(2)	
	13	178.00	163.00	188.00	211.50	165.50	N
					3.536	20.506	
		(1)	(1)	(1)	(2)	(2)	
	32	248.00	188.00	229.00	225.00	216.00	N
					32.527	4.243	
		(1)	(1)	(1)	(2)	(2)	
Activated Partial Thromboplastin Time .....(sec)	-2	20.70	21.75	21.25	21.46	20.32	P
		2.825	0.545	3.131	2.844	3.283	
		(4)	(4)	(4)	(5)	(5)	

(Females - continue)		Gr# 1	Gr# 2	Gr# 3	Gr# 4	Gr# 5	ANOVA
Dose (mg/kg/week)		0	5	25	125	75	
	Week						
							P
Activated Partial Thromboplastin Time .....(sec)	6	19.10	21.27	19.95	20.08	19.08	
		1.421	2.081	3.025	2.158	2.179	
		(4)	(4)	(4)	(5)	(5)	
							N
	8	18.00	22.50	26.10	21.00	18.95	
					0.000	2.051	
		(1)	(1)	(1)	(2)	(2)	
							N
	13	19.60	21.00	22.60	19.95	18.85	
					0.919	3.041	
		(1)	(1)	(1)	(2)	(2)	
							N
	32	15.60	20.30	20.10	17.80	16.10	
					0.424	1.697	
		(1)	(1)	(1)	(2)	(2)	

ANOVA: "P" = Parametric; "N" = Non Parametric; "-" = Not processed
Significance Level: * p < 0.05; ** p < 0.01; *** p < 0.001

5.2.2 Blood Chemistry

Compared to the control group, some treatment-related changes in blood chemistry tests appeared in the animals treated by either intravenous or subcutaneous route at the end of the 5-week treatment ***(Tables 5 and 6)***.

Minor decreases in IgG and IgM were detected at the two higher IV doses and in the group treated by SC route at the end of the dosing period but of no biological meaning.

Several changes were observed in both sexes treated by either intravenous or subcutaneous (only for one female animal) route. However, all the findings in alpha 1 glycoprotein acid and C-reactive protein were considered transient changes, being dose-unrelated or time-unrelated.

Table 5: Blood chemistry relevant parameters - Males (monkeys)
(Mean, S.D., n)

Dose (mg/kg/week)	Week	Gr# 1 0	Gr# 2 5	Gr# 3 25	Gr# 4 125	Gr# 5 75	ANOVA
Alpha 1 Glycoprotein Acid .....(mg/dL)	-2	34.00 10.739 (4)	n.d.	23.00 (1)	22.00 0.000 (2)	24.50 2.646 (4)	N
	1	30.75 8.421 (4)	23.67 6.351 (3)	25.25 3.202 (4)	36.20 23.317 (5)	30.50 6.557 (4)	P
	3	35.50 4.950 (2)	26.50 0.707 (2)	25.00 2.828 (2)	28.00 9.644 (3)	26.75 7.890 (4)	P
	3	33.50 4.950 (2)	28.00 6.557 (3)	24.50 0.707 (2)	26.00 6.377 (4)	28.00 7.165 (4)	P
	5	35.00 4.243 (2)	26.50 7.778 (2)	24.67 6.429 (3)	28.67 10.786 (3)	30.00 1.414 (2)	P
	5	28.50 7.506 (4)	27.67 11.590 (3)	27.75 7.411 (4)	32.00 13.323 (5)	33.33 9.713 (3)	P
	6	36.00 11.518 (4)	24.50 6.364 (2)	24.00 5.292 (3)	27.60 8.204 (5)	28.25 8.655 (4)	P
Alpha 1 Glycoprotein Acid .....(mg/dL)	8	20.00 (1)	23.00 (1)	n.d.	29.50 12.021 (2)	32.50 0.707 (2)	N
	13	n.d.	n.d.	n.d.	n.d.	27.00 2.828 (2)	-
	32	57.00 (1)	n.d.	21.00 (1)	20.00 (1)	26.00 2.828 (2)	N
Immunoglobulin G .....(mg/dL)	-2	1231.50 67.456 (4)	1284.75 278.620 (4)	1006.00 127.088 (4)	1230.00 147.907 (5)	1149.60 155.146 (5)	P
	6	1227.50 124.551 (4)	1278.50 253.728 (4)	957.75 171.165 (4)	1113.40 240.242 (5)	948.00 205.359 (5)	P
	8	1087.00 (1)	1478.00 (1)	720.00 (1)	1216.00 642.053 (2)	1135.50 327.390 (2)	N
	13	1065.00 (1)	1198.00 (1)	791.00 (1)	1201.00 200.818 (2)	1161.50 184.555 (2)	N
	32	970.00 (1)	1055.00 (1)	831.00 (1)	1019.00 101.823 (2)	1046.00 114.551 (2)	N
Immunoglobulin M .....(mg/dL)	-2	101.50 30.946 (4)	91.25 38.483 (4)	91.75 26.538 (4)	88.40 26.159 (5)	94.80 22.499 (5)	P

(Males - continue)		Gr# 1	Gr# 2	Gr# 3	Gr# 4	Gr# 5	ANOVA
Dose (mg/kg/week)		0	5	25	125	75	
	Week						
							P
Immunoglobulin M .....(mg/dL)	6	100.50	71.50	89.25	63.40	60.00	
		33.491	9.883	31.009	28.059	10.607	
		(4)	(4)	(4)	(5)	(5)	
							N
	8	95.00	57.00	75.00	43.50	59.50	
					6.364	4.950	
		(1)	(1)	(1)	(2)	(2)	
							N
	13	83.00	55.00	71.00	53.00	66.00	
					14.142	21.213	
		(1)	(1)	(1)	(2)	(2)	
							N
	32	80.00	48.00	52.00	36.00	53.00	
					7.071	1.414	
		(1)	(1)	(1)	(2)	(2)	
							P
C-Reactive protein .....(mg/L)	-2	2.30	2.27	0.71	0.84	1.08	
		2.706	1.854	0.364	0.567	1.010	
		(4)	(4)	(4)	(5)	(5)	
							P
	1	2.06	3.59	1.84	6.53	1.06	
		1.461	3.104	2.049	11.277	0.475	
		(4)	(4)	(4)	(5)	(5)	
							P
	3	1.20	4.00	1.19	1.22	1.03	
		0.753	2.882	0.971	0.583	0.512	
		(4)	(4)	(4)	(5)	(5)	
							N
	3	1.24	6.15	1.39	1.17	1.08	
		0.289	6.956	1.180	0.407	0.562	
		(4)	(4)	(4)	(5)	(5)	
							P
	5	1.12	2.87	1.48	2.17	0.88	
		0.508	2.331	0.950	2.149	0.526	
		(4)	(4)	(4)	(5)	(5)	
							P
	5	1.32	4.35	1.41	2.45	1.08	
		0.598	4.888	0.579	1.240	0.460	
		(4)	(4)	(4)	(5)	(5)	
							P
	6	1.30	1.37	0.88	1.42	0.92	
		1.281	0.744	0.863	1.054	0.753	
		(4)	(4)	(4)	(5)	(5)	
							N
	8	0.87	2.46	0.89	1.94	0.97	
					1.690	0.566	
		(1)	(1)	(1)	(2)	(2)	
							N
	13	0.65	2.85	0.56	1.35	0.98	
					0.516	0.912	
		(1)	(1)	(1)	(2)	(2)	
							N
	32	0.40	1.99	2.67	1.52	0.95	
					0.064	0.863	
		(1)	(1)	(1)	(2)	(2)	

ANOVA: "P" = Parametric; "N" = Non Parametric; "-" = Not processed
Significance Level: * p < 0.05; ** p < 0.01; *** p < 0.001

Table 6: Blood chemistry relevant parameters - Females (monkeys)
(Mean, S.D., n)

Dose (mg/kg/week)	Week	Gr# 1 0	Gr# 2 5	Gr# 3 25	Gr# 4 125	Gr# 5 75	ANOVA	
Alpha 1 Glycoprotein Acid .....(mg/dL)	-2	31.67 14.154 (3)	26.00 7.211 (3)	25.75 7.136 (4)	23.67 1.155 (3)	25.75 6.551 (4)	P	
	1	30.75 11.587 (4)	32.00 11.916 (4)	35.50 13.916 (4)	38.20 9.731 (5)	47.60 35.872 (5)	P	
	3	32.67 17.616 (3)	31.33 8.622 (3)	27.67 4.041 (3)	25.00 2.345 (5)	30.50 9.815 (4)	P	
	3	35.67 14.154 (3)	29.75 8.057 (4)	28.25 6.238 (4)	25.60 2.966 (5)	30.50 7.188 (4)	P	
	5	37.75 14.245 (4)	88.00 97.041 (3)	27.33 1.528 (3)	26.60 3.286 (5)	24.75 1.708 (4)	N	*
	5	37.75 15.019 (4)	107.75 154.957 (4)	29.75 7.588 (4)	37.20 4.817 (5)	26.60 5.079 (5)	P	
	6	35.50 17.464 (4)	51.50 48.473 (4)	29.33 7.572 (3)	29.80 4.970 (5)	24.50 2.380 (4)	P	
	8	20.00 (1)	21.00 (1)	26.00 (1)	21.00 0.000 (2)	25.00 1.414 (2)	N	
	13	24.00 (1)	n.d. 	22.00 (1)	24.00 (1)	23.50 0.707 (2)	N	
	32	32.00 (1)	n.d. 	25.00 (1)	20.00 (1)	22.50 0.707 (2)	N	
Immunoglobulin G .....(mg/dL)	-2	1342.25 65.688 (4)	1172.75 83.252 (4)	1151.75 256.904 (4)	1140.20 107.855 (5)	1179.80 122.757 (5)	P	
	6	1219.00 91.240 (4)	1087.50 73.822 (4)	930.75 268.891 (4)	836.20 202.355 (5)	912.60 237.596 (5)	P	
	8	1227.00 (1)	1201.00 (1)	839.00 (1)	960.50 253.851 (2)	949.00 113.137 (2)	N	
	13	1308.00 (1)	1305.00 (1)	872.00 (1)	1165.00 164.049 (2)	1142.50 27.577 (2)	N	
	32	1298.00 (1)	1182.00 (1)	813.00 (1)	1085.50 228.395 (2)	1105.00 96.167 (2)	N	
Immunoglobulin M .....(mg/dL)	-2	116.75 24.171 (4)	89.00 14.810 (4)	78.75 27.256 (4)	78.80 18.660 (5)	93.20 41.746 (5)	P	

(Females - continue)		Gr# 1	Gr# 2	Gr# 3	Gr# 4	Gr# 5	ANOVA
Dose (mg/kg/week)		0	5	25	125	75	
	Week			*	**	*	P **
Immunoglobulin M .....(mg/dL)	6	104.75	77.50	53.25	37.60	61.40	
		24.865	16.523	18.715	8.503	31.230	
		(4)	(4)	(4)	(5)	(5)	
							N
	8	97.00	94.00	41.00	31.50	41.50	
					0.707	0.707	
		(1)	(1)	(1)	(2)	(2)	
							N
	13	96.00	94.00	52.00	44.50	48.50	
					7.778	9.192	
		(1)	(1)	(1)	(2)	(2)	
							N
	32	102.00	72.00	173.00	52.50	56.50	
					2.121	2.121	
		(1)	(1)	(1)	(2)	(2)	
							P
C-Reactive protein .....(mg/L)	-2	1.14	1.11	2.50	0.98	1.73	
		0.668	0.408	3.488	0.451	1.582	
		(4)	(4)	(4)	(5)	(5)	
							P
	1	1.19	1.43	2.61	5.49	7.61	
		0.638	0.989	3.171	2.867	9.231	
		(4)	(4)	(4)	(5)	(5)	
							P
	3	1.24	1.08	1.32	1.60	1.42	
		0.691	0.654	1.006	0.873	1.041	
		(4)	(4)	(4)	(5)	(5)	
							P
	3	1.36	1.40	1.81	1.56	1.37	
		0.443	0.589	1.200	0.819	1.017	
		(4)	(4)	(4)	(5)	(5)	
							P
	5	2.78	7.97	1.27	2.30	1.36	
		3.200	13.700	0.880	1.758	1.001	
		(4)	(4)	(4)	(5)	(5)	
							P
	5	1.37	7.11	1.98	4.19	1.97	
		0.622	11.025	1.228	3.999	1.469	
		(4)	(4)	(4)	(5)	(5)	
							P
	6	1.54	1.33	1.26	1.49	1.16	
		0.815	0.565	1.215	0.740	1.004	
		(4)	(4)	(4)	(5)	(5)	
							N
	8	0.51	0.56	0.28	0.63	0.65	
					0.523	0.028	
		(1)	(1)	(1)	(2)	(2)	
							N
	13	0.41	0.27	0.11	0.83	1.18	
					0.905	1.195	
		(1)	(1)	(1)	(2)	(2)	
							N
	32	4.89	0.62	0.18	1.84	1.48	
					0.813	1.435	
		(1)	(1)	(1)	(2)	(2)	

ANOVA: "P" = Parametric; "N" = Non Parametric; "-" = Not processed
Significance Level: * p < 0.05; ** p < 0.01; *** p < 0.001

5.2.3 Urinalysis

No treatment-related variations in urinalyses were noted in any animal treated at various dosages by either intravenous or subcutaneous route at the end of the 5-week treatment period or at any test point during the recovery period.

5.3 PATHOLOGY

5.3.1 Organ weights

No organ weight variations related to treatment were seen at either sacrifice.

5.3.2 Macroscopic Findings

No treatment-related changes were noted at either sacrifice.

At final killing, sporadic cases of perivascular hemorrhage were seen at the injection site of control and treated animals and considered to be caused by needle trauma.

5.3.3 Microscopic Findings

No systemic changes were seen in animals treated with Anti-CD25 by intravenous or subcutaneous routes at the final sacrifice.
At the injection site, treatment-related changes were seen only in animals injected subcutaneously with 75 mg/kg/day (group 5) of the test item. Such changes consisted of minimal or mild perivascular mononuclear cell infiltration.
A range of lesions was seen in several organs, all considered as incidental and of no relation to treatment. In fact, such changes had a comparable incidence in control and treated groups, or are characteristically seen in control Cynomolgus monkeys used in our laboratory. In particular, presence of hemorrhage, subcutaneous and vascular hemorrhage at the injection sites had a comparable incidence in control and treated animals and were considered to be related to needle trauma. After the withdrawal period, local changes induced by subcutaneous treatment had recovered completely.

5.4 TOXICOKINETICS

Anti-CD25 mAb was administered once a week for 5 consecutive weeks to Cynomolgus monkeys. Three groups were treated by intravenous route at 5 mg/kg (4 males and 4 females), at 25 mg/kg (4 males and 4 females) and at 125 mg/kg (5 males and 5 females). The fourth group was treated by subcutaneous route at 75 mg/kg (5 males and 5 females). The control group (4 males and 4 females) received 0 mg/kg dose (vehicle) by intravenous route.

Complete toxicokinetic profiles were monitored after the first (Day 1) and the last treatment (Day 29). The following samplings were carried out also during the intermediate period: at all doses IV: 2nd, 3rd and 4th treatment, times 0 and 0.5 hours; SC 2nd, 3rd and 4th treatment, times 0 and 4 hours. Sampling was performed also in recovery animals (1 male and 1 female at 5 and 25 mg/kg IV doses; 2 males and 2 females at 125 mg/kg IV dose and 75 mg/kg SC dose). All the animals were exposed to the test item for the entire treatment periods. No test item amounts were found in animals at pre-dose sampling.
The main PK parameters are reported in the following table as mean ± S.D. values (***Table 7***).

Table 7: PK parameters - (Mean ± S.D.)

Dose (mg/kg)		**5**	**25**	**125**
Dose ratio		**1**	**5**	**25**
INTRAVENOUS ADMINISTRATION				
Day 1				
C_{max}	(µg/mL)	132.0 ± 39.5	522.2 ± 92.3	3120 ± 1954
t_{max} (1)	(h)	0.083 (0.083-3)	0.083 (0.083-0.5)	0.29 (0.083-3)
AUC_{0-168}	(h·µg/mL)	5853 ± 789	25511 ± 4185	141243 ± 22434
AUC	(h·µg/mL)	8675 ± 1529	39850 ± 11861	231234 ± 60164
MRT_{168}	(h)	64.3 ± 1.6	66.1 ± 5.0	65.9 ± 3.4
$t_{1/2}$	(h)	100.9 ± 21.7	106.1 ± 40.1	121.9 ± 55.7
Cl	(mL/h/kg)	0.60 ± 0.12	0.67 ± 0.17	0.57 ± 0.13
Vz	(mL/kg)	84.36 ± 13.52	97.05 ± 30.81	92.81 ± 21.13
Vss	(mL/kg)	84.58 ± 10.81	99.31 ± 23.58	93.45 ± 19.78
DP AUC_{0-168} ratio			4.4	24.1

Day 29							
			**		**		**
C_{max}	(µg/mL)	207.2 ± 79.4		898.8 ± 304.9		4752 ± 4277	
t_{max} (1)	(h)	0.5 (0.083-24)		0.5 (0.083-4)		0.5 (0.083-3)	
AUC_{0-168}	(h·µg/mL)	12849 ± 3710		46887 ± 23191		230391 ±51877	
AUC_z	(h·µg/mL)		27178		99093		559334 ± 140592
AUC	(h·µg/mL)	17689 ± 3784	27245	86682 ± 48017	99106	342852±59303	559414 ± 140634
MRT_{168}	(h)	68.6 ± 2.1		67.7 ± 4.7		65.7 ± 3.2	
MRT_z	(h)		218. 3		221.4		371.9 ± 35.0
$t_{1/2}$	(h)	118.5 ± 35.6	201.3	120.6 ± 40.3	180.5	103.7 ± 15.1	265.0 ± 40.4
Cl	(mL/h/kg)	0.30 ± 0.06	0.19	0.34 ± 0.13	2.33	0.38 ± 0.07	0.24 ± 0.07
Vz	(mL/kg)	47.91 ± 5.36	52.60	53.71 ± 9.43	109.30	55.29 ± 8.23	91.41 ± 34.68
Vss	(mL/kg)	48.19 ± 3.42	39.97	55.07 ± 9.00	87.16	55.13 ± 8.82	89.50 ± 35.45
DP AUC_{0-168} ratio				3.7		17.9	
R_{ac}		2.3 ± 1.0		1.8 ± 0.8		1.6 ± 0.3	
AUCτ|-AUC∞□ratio		1.58		1.15		1.04	

Dose (mg/kg)		**75**		
SUBCUTANEOUS ADMINISTRATION				
		Day 1	**Day 29**	
				**
C_{max}	(µg/mL)	980.7 ± 913.7	1965 ± 2249	
t_{max} (1)	(h)	24 (24-24)	24 (6-432)	
AUC_{0-168}	(h·µg/mL)	82589 ± 33529	157484 ± 43862	
AUC_z	(h·µg/mL)			401483 ± 106974
AUC	(h·µg/mL)	157682 ± 46128	323820 ± 106009	401509 ± 106998
$t_{1/2}$	(h)	153. 0 ± 83.4	165.1 ± 43.4	236.6 ± 114.0
MRT_{168}	(h)	74.2 ± 7.6	72.9 ± 6.2	
MRT_z	(h)			375.6 ± 95.4
R_{ac}			2.1 ± 0.8	
F		1.08	1.12	
AUCτ-AUC∞□ ratio			1.08	

* As regards Day 29, group 2, subject no. 311021M was excluded from mean ± SD calculation

** Values derived from animals undergoing recovery period

(1) Median and range are indicated for tmax

DP AUC0-168 ratio = Dose proportionality, calculated by AUC0-168h (Dose 25 or 125 mg/kg)/ AUC0-168 (Dose 5 mg/kg) values

Rac = Accumulation ratio, calculated by AUC0-168h (Day 29)/ AUC0-168 (Day 1) values

F= Absolute bioavailability calculated by AUC0-168h (s.c) vs. AUC0-168h (i.v., dose 25 mg/kg) values

Absorption rate:
Serum levels were seen to increase after IV doses

Dose proportionality:
The compound was quantifiable at all sampling times in all groups, with good dose proportionality. On Day 29 the increase in exposure remained still roughly proportional with the dose. As expected, AUC values were higher in animals following recovery period, confirm the acceptable dose proportionality. After subcutaneous dose, it was showed an increased exposure between the first and the last, 5th, administration.

Mean residence time:
MRT values (after IV doses till 168 h) were comparable between sampling days and overall were about 3 days. Longer MRT were observed in recovery animals (range 10-15 days). MRT values found after SC administrations were similar to those after IV treatments, being about 3 days on both Day 1 and Day 29 and about 15 days in the animals under recovery monitoring.

Half-life values:
Animals treated by IV route showed a very slow elimination rate in the 0- 168 h interval; half-life values, on Day 1 ranging from 101 h (about 4 days) to122 h (about 5 days) and 104–121 h (about 4-5 days) on Day 29. A longer half–life was observed after the last dose in recovery animals, ranging from 180 to 265 h (about 7.5-11 days). Also SC administration showed slow compound elimination. Half-life was 153 h (about 6 days) and 165 h (about 7 days) when calculated until 168 h on Day 1 and Day 29 and 237 h (about 10 days) in recovery animals. These values are similar to those obtained after IV treatments.

Clearance and Distribution volume:
PK profiles followed until 168 h showed low clearance values (range 0.57-0.67 mL/h/kg) on Day 1. Distribution volume values at steady state, Vss, were in general low at all doses and sampling periods. On Day 1 they ranged 84.6-99.3 mL/kg and on Day 29 ranged 48.2-55.1 mL/kg. As regard recovery animals, they showed low Vss values, namely in the range from 40.0 to 89.5 mL/kg.

The results suggested compound distribution limited to the volume of the extracellular space, due to high molecular weight and therefore low ability to cross membranes.

Accumulation ratio:
Compound accumulation was estimated by Rac values, calculated as the AUC0-168h Day 29/AUC0-168h Day 1 ratio. As general trend, at low doses there was higher accumulation, but also higher data variability (as expected). Accumulation was not overall noticed by either IV or IV treatments, with the exception of group 2 (ratio=1.6 and Rac=2.3), anyway the values were not however statistically significant (Student T test= 0.06).

To better clarify the matter, concentration at steady state was calculated using WinNonLin (nonparametric superposition tool), the results are reported in the following table (***Table 8***) and are consistent with the above conclusions.

Table 8: Compound accumulation ratio

Group 2 (IV, 5 mg/kg)			
Animal n°	AUC_{0-168} (5th adm)	AUCinf (1st adm)	Ratio
212101M	11660	9731	1.20
309087M	10684	6646	1.61
401043M	20336	6299	3.23
312224F	12113	10255	1.18
312342F	10367	8278	1.25
401126F	9811	10240	0.96
401208F	14974	9303	1.61
		Mean	1.58
		SD	0.77
		CV	49%

p=0.059§

Group 3 (IV, 25 mg/kg)			
Animal n°	AUC_{0-168} (5th adm)	AUCinf (1st adm)	Ratio
208097M	35163	31414	1.12
209075M	42885	37880	1.13
310153M	45806	36678	1.25
402027M	5469	31495	0.17
109110F	82667	64900	1.27
304172F	49243	42980	1.15
312398F	42667	27425	1.56
312490F	71199	46029	1.55
		Mean	1.15
		SD	0.43
		CV	38%

p=0.233§

*: Animal 311021M excluded due to a possible administration error.

Group 4 (IV, 125 mg/kg)			
Animal n°	AUC_{0-168} (5th adm)	AUCinf (1st adm)	Ratio
10103M	264142	209180	1.26
308363M	199081	229522	0.87
309073M	182567	337959	0.54
310411M	213797	280011	0.76
404015M	205143	210721	0.97
306520F	264044	192889	1.37
307610F	224675	191894	1.17
308074F	238638	172798	1.38
312210F	176587	168998	1.04
402134F	337677	318366	1.06
		Mean	1.04
		SD	0.27
		CV	26%

p=0.979§

Group 5 (SC, 75 mg/kg)			
Animal n°	AUC_{0-168} (5th adm)	AUCinf (1st adm)	Ratio
202205M	169485	191191	0.89
210253M	155849	198028	0.79
309365M	159328	113267	1.41
312003M	202331	131099	1.54
401037M	130459	131293	0.99
108190F	148423	86972	1.71
302688F	107179	160352	0.67
303226F	254907	168332	1.51
307140F	122713	150033	0.82
401222F	124162	246251	0.50
		Mean	1.08
		SD	0.42
		CV	39%

p=0.993§

§ Student t-test, paired data.

Absolute subcutaneous bioavailability:
The F values, calculated from the ratio of AUC0-168h on Day 1 or Day 29 vs. the AUC0-168h found at 25 mg/kg IV, were 1.08 after the first administration and 1.12 after the last (5th) treatment, indicating a good compound bioavailability by this route at 75 mg/kg. No gender difference was found.

Toxicokinetics evaluation:
Serum level increased between the first and the last treatment at all doses by both administration routes. Evaluations were carried out on data from all animals on Day 1 (first treatment) and on Day 29 (last, 5th treatment) until the 168 h sampling time after both administration routes, then extended to recovery animal data. After intravenous doses, concentrations peaked at 0.083-0.5 h, increasing between the sampling periods. Acceptable dose proportionality was found.
Compound residence in the body, evaluated as MRT, was long, about 3 days on both Day 1 and Day 29, increasing to 10- 15 days in animals during the recovery period. Therefore and as expected, also elimination occurred at very slow rate, in general recording a half-life of about 4-5 days on Day 1 and Day 29 or longer, 10-15 days, in the profiles of animals that completed kinetics during the recovery period. Low clearance and small distribution volume at steady-state were found. In general their values were seen to decrease from Day 1 to Day 29 and to recovery weeks, suggesting compound distribution in the extracellular fluids. Accumulation was not observed at any dose after intravenous dosing.
Subcutaneous treatment attained their maxima levels usually within 24 h and concentrations increased between the first (Day 1) and the last (Day 29) administration. Exposure increased with time. The long mean residence time, about 3 days on Day 1 and Day 29, was still longer, about 15 days, in recovery animals. Similar values were recorded after IV doses. Half-life values were 6-7 days on Day 1 - Day 29 and about 10 days in recovery animals. No significant compound accumulation was found after multiple SC administration. Bioavailability was 108% after the first treatment and 112% after the last treatment.

5.5 PD MARKERS: LEUKOCYTE AND LYMPHOCYTE SUBSETS; CD25 EXPRESSION, SATURATION AND DOWN-MODULATION ON LYMPHOCYTE SUBSETS

CD25 expression, saturation and down modulation

CD25 expression on T cytotoxic cells was lower than 1% over time.

Therefore, data related to this lymphocyte subpopulation were not reported. Because CD25 expression was limited to CD4+ T-cells, the analyses of CD25 expression, saturation by Anti-CD25 (***Figure 43 and 44***) and CD25 down-modulation were focused on this population only.

CD25 expression, saturation

Spontaneous expression of CD25 antigen in untreated healthy Cynomolgus monkey was detected on about 4% of CD4+ T-cells (mean from pre-trial data of 44 animals, 22M + 22F).

IL-2Rα saturation was recorded at 3 and 6 h after Anti-CD25 administered by iv route at 5, 25, 125 mg/kg and by sc route at 75 mg/kg, respectively, confirming data obtained in previous experiments.
This parameter was evaluated once a week, before each treatment until the end of Anti-CD25 administration (Day 29).

CD25 saturation was observed in all dosed animals until the end of treatment (Day 28) with the exception of two male animals (Group 2 and (Group 3) in which CD25 expression near to pre-treatment value (2.1 and 3.2%, respectively), started to show from Day 14 and at the end of treatment, respectively.

CD25 desaturation

CD25 desaturation, detected by anti-CD25/ACT-1 binding, was measured at biweekly intervals in the first 6 weeks after treatment and at monthly intervals until complete desaturation was reached in 1 animal/sex in groups 2 and 3, and 2 monkeys/sex in groups 4 and 5.

CD25 desaturation was defined for each treated animal as return to a percentage of CD25+ cells similar to the pre-treatment value, as well as maintenance of constant values of CD25+ cell percentage.

Moreover, for all animals undergoing recovery, CD25 desaturation was assessed as absence of Anti-CD25 bound to CD4+ T-cells detected with an anti-human IgG PE-conjugated mAb able to bind Anti-CD25 on the cell surface.

CD25 desaturation was reached in a dose-dependent manner with the exception of the animal dosed at 25 mg/kg by IV route, in which the recovery of CD25 was observed on Day 28.

Moreover the period to achieve the CD25 expression in female animals was longer than in male animals as reported below:

Male animals
- at week 6 of recovery in animals dosed at 5 mg/kg by IV route;
- at Day 28 of treatment in animals dosed at 25 mg/kg by IV route;

- between weeks 11-18 of recovery in animals dosed at 125 and 75 mg/kg by iv and SC route, respectively.

Female animals

- at week 14 of recovery in animals dosed at 5 mg/kg by IV route;
- at week 23 of recovery in animals dosed at 25 mg/kg by IV route;
- at week 29 of recovery in animals dosed at 125 and 75 mg/kg by IV and SC route, respectively.

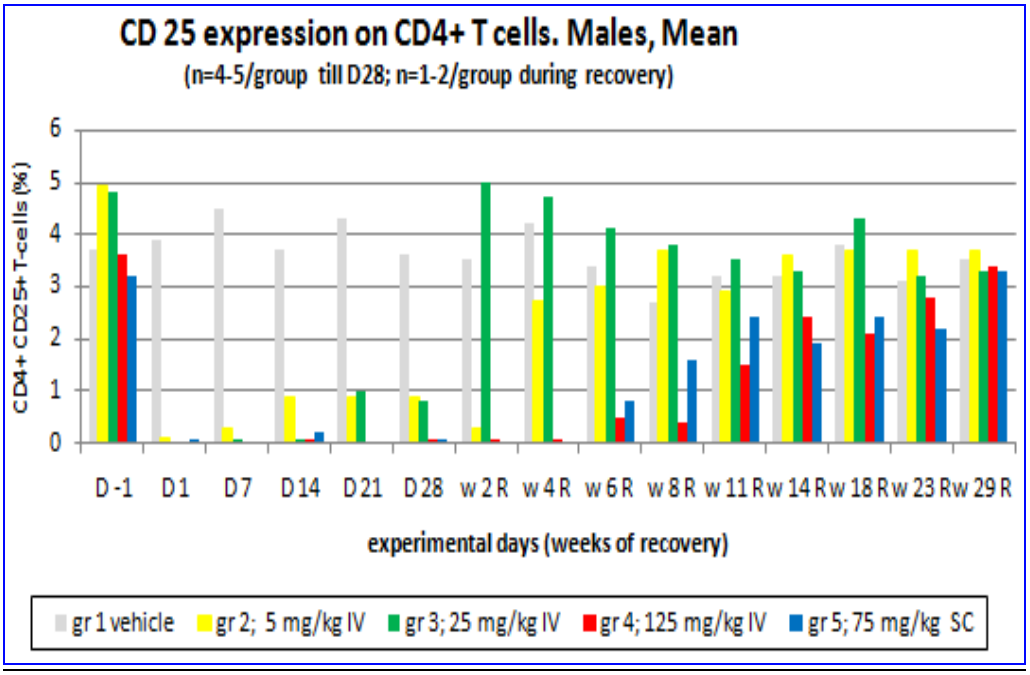


Fig. 43: *CD25 expression and saturation on CD4+ T cells - Males (monkeys)*

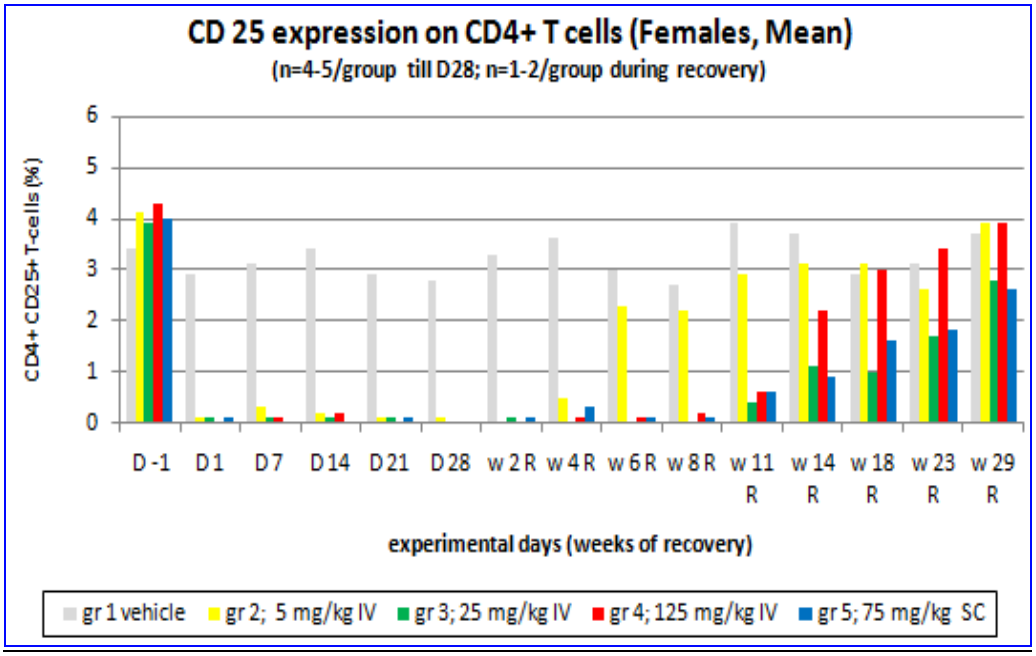


Fig.44: *CD25 expression and saturation on CD4+ T cells - Females (monkeys)*

CD25 down-modulation

A trend toward CD25 down-modulation was observed at all doses (***Figures 45 and 46***).

A statistically significant reduction in CD25+ cell % was recorded from Day 7 to Day 28 in animals dosed at 125 mg/kg by IV route and on Days 14 and 28 in animals dosed at 75 mg/kg by SC route.

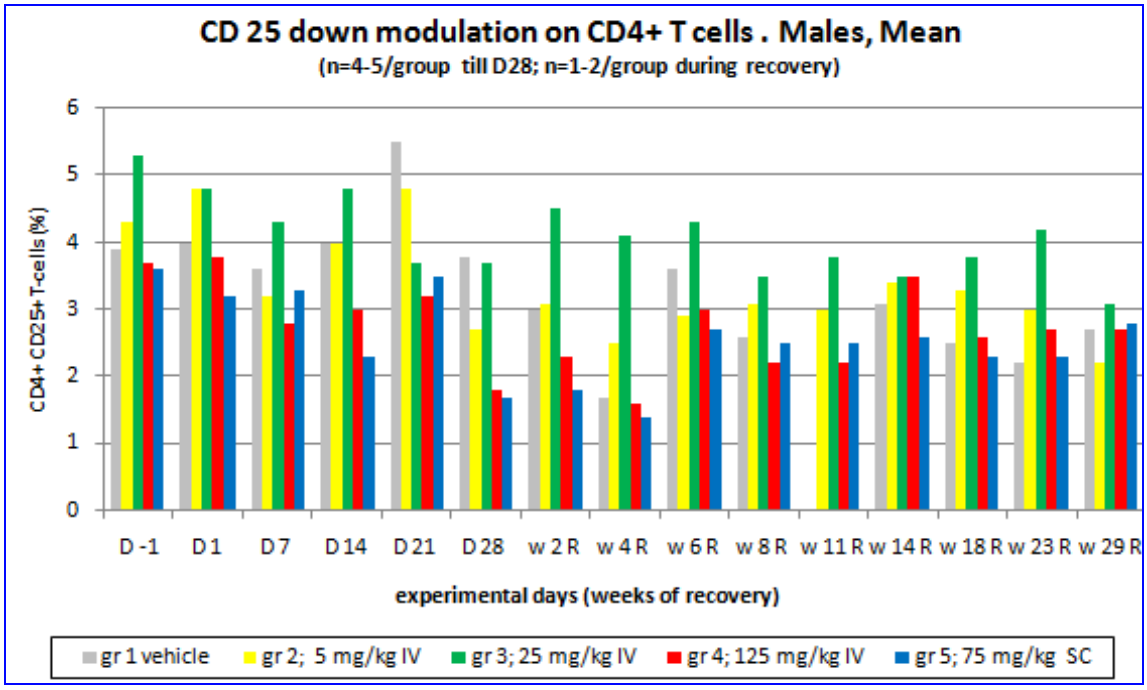


Fig. 45:*CD25 down modulation on CD4+ T cells – Males (monkeys)*

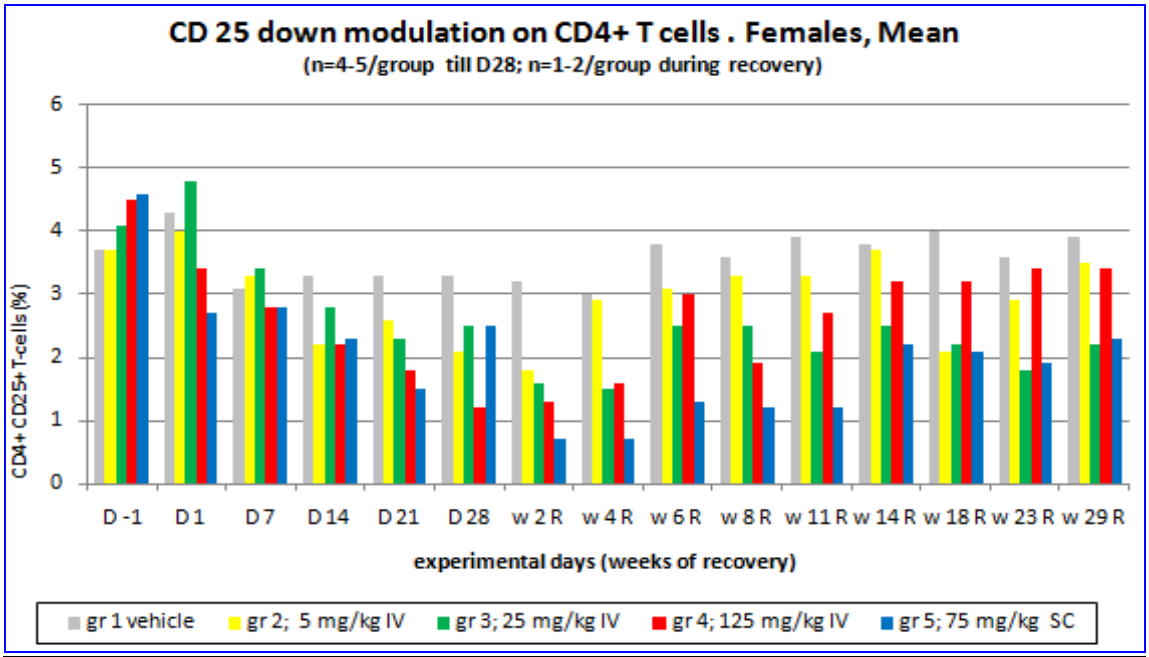


Fig.46: *CD25 down modulation on CD4+ T cells – Females (monkeys)*

Total leukocytes, leukocyte subsets and lymphocyte subsets

Individual data of leukocytes, leukocyte subsets (lymphocytes and neutrophils, absolute count) monocytes and lymphocyte subpopulations absolute counts are reported (***Table 9***).
No treatment-related effects were seen on total leukocytes and leukocyte subsets, on total Tcells, helper T cells, cytotoxic T-cells, B-cells and NK-cells over time.
A statistically significant increase in leukocyte and neutrophils absolute counts (***Table 10***), observed 3-6 hours after the beginning of treatment, seems to be related to the frequent blood sampling performed during the study to carry out the different analyses scheduled.
Other statistically significant variations observed in some lymphocyte subsets reported as absolute counts, were considered treatment-unrelated and without any biological relevance.

Table 9: Lymphocyte subsets and Monocytes absolute values - (Mean ± S.D.)

Group 1 (IV) - 0 mg/kg - Lymphocyte subsets and Monocytes (cells/µL of blood) - ***Males***

Sampling Time		D1(pre-treat)	D1(after 3 hrs)	D7	D14	D21	D28
T cells	Mean	4352	2505	4344	4847	4557	3799
	S.D.	990	424	1936	3274	3268	1189
	N	4	4	4	4	4	4
T helper cells	Mean	2270	1403	2021.75	2235	2124.25	1855
	S.D.	665	392	702	1147	1522	465
	N	4	4	4	4	4	4
T cytotoxic cell	Mean	2095	1100	2346	2597	2420	1945
	S.D.	788	47	1556	2459	2260	994
	N	4	4	4	4	4	4
B cells	Mean	2228	1866	2134	2365	1924	2129
	S.D.	1170	1300	836	609	491	1058
	N	4	4	4	4	4	4
NK cells	Mean	875	339	540	447	442	773
	S.D.	79	177	347	325	462	285
	N	4	4	4	4	4	4
Monocyte	Mean	640	841	595	596	471	468
	S.D.	81	155	164	376	265	216
	N	4	4	4	4	4	4

Group 1 (IV) - 0 mg/kg - Lymphocyte subsets and Monocytes (cells/µL of blood) - ***Females***

Sampling Time		D1(pre-treat)	D1(after 3 hrs)	D7	D14	D21	D28
T cells	Mean	5167	3802	3739	4183	3784	4454
	S.D.	2062	1910	2182	1667	1359	1884
	N	4	4	4	4	4	4
T helper cells	Mean	2687	2269	2095	2186	2082	2309
	S.D.	721	846	892	629	587	675
	N	4	4	4	4	4	4
T cytotoxic cell	Mean	4548.3	1534.5	1648.5	2011.5	1711.3	2131.3
	S.D.	3584	1118	1280	1042	774	1149
	N	4	4	4	4	4	4
B cells	Mean	1364	1608	1250	1268	1236	1247
	S.D.	358	431	366	243	250	364
	N	4	4	4	4	4	4
NK cells	Mean	798	431	565	672	603	926
	S.D.	727	484	466	398	297	408
	N	4	4	4	4	4	4
Monocyte	Mean	865	915	586	568	395	984
	S.D.	96	587	247	321	153	947
	N	4	4	4	4	4	4

Group 2 (IV) - 5 mg/kg - Lymphocyte subsets and Monocytes (cells/μL of blood) - ***Males***

Sampling Time		D1(pre-treat)	D1(after 3 hrs)	D7	D14	D21	D28
T cells	Mean	3962	2994	3803	3551	3983	4092
	S.D.	1447	1851	1567	1684	2031	770
	N	4	4	4	4	4	4
T helper cells	Mean	2233	1720	2089	2056	2260	2260
	S.D.	730	895	784	867	1130	154
	N	4	4	4	4	4	4
T cytotoxic cell	Mean	1719	1262	1709	1491	1715	1837
	S.D.	735	944	792	835	891	650
	N	4	4	4	4	4	4
B cells	Mean	1491	1278	1380	1283	1329	1519
	S.D.	751	781	661	729	458	551
	N	4	4	4	4	4	4
NK cells	Mean	900	462	834	688	724	762
	S.D.	112	154	145	166	118	226
	N	4	4	4	4	4	4
Monocyte	Mean	461	702	555	799	394	627
	S.D.	221	370	228	603	228	451
	N	4	4	4	4	4	4

Group 2 (IV) - 5 mg/kg - Lymphocyte subsets and Monocytes (cells/μL of blood) - ***Females***

Sampling Time		D1(pre-treat)	D1(after 3 hrs)	D7	D14	D21	D28
T cells	Mean	6384	3312	4342	4507	3480	4253
	S.D.	2326	472	1642	1301	1341	2632
	N	3	4	4	4	4	4
T helper cells	Mean	3795	2046	2554	2769	2149	2628
	S.D.	1874	300	1520	1203	1039	1941
	N	3	4	4	4	4	4
T cytotoxic cell	Mean	2577	1262	1623	1729	1335	1636
	S.D.	443	323	566	147	308	709
	N	3	4	4	4	4	4
B cells	Mean	1595	1333	1312	1300	1109	1185
	S.D.	538	303	700	491	406	786
	N	3	4	4	4	4	4
NK cells	Mean	1104	406	785	723	835	1049
	S.D.	993	175	268	260	395	355
	N	3	4	4	4	4	3
Monocyte	Mean	900	1043	1027	713	688	1380
	S.D.	212	617	365	112	318	1494
	N	3	4	4	4	4	4

Group 3 (IV) - 25 mg/kg - Lymphocyte subsets and Monocytes (cells/µL of blood) - ***Males***

Sampling Time		D1(pre-treat)	D1(after 3 hrs)	D7	D14	D21	D28
T cells	Mean	2817	2451	2519	2455	2483	2695
	S.D.	663	572	546	391	255	934
	N	4	4	4	4	4	4
T helper cells	Mean	1787	1521	1582	1542	1479	1625
	S.D.	465	240	348	220	158	589
	N	4	4	4	4	4	4
T cytotoxic cell	Mean	1032	932	945	918	1011	1070
	S.D.	249	338	257	268	213	397
	N	4	4	4	4	4	4
B cells	Mean	1196	1205	1198	1135	1205	1326
	S.D.	457	434	385	357	392	493
	N	4	4	4	4	4	4
NK cells	Mean	971	504	899	804	825	768
	S.D.	487	301	254	240	188	219
	N	4	4	4	4	4	4
Monocyte	Mean	807	748	356	395	236	535
	S.D.	309	414	198	201	175	409
	N	4	4	4	4	4	4

Group 3 (IV) - 25 mg/kg - Lymphocyte subsets and Monocytes (cells/µL of blood) - ***Females***

Sampling Time		D1(pre-treat)	D1(after 3 hrs)	D7	D14	D21	D28
T cells	Mean	5925	2705	4790	4670	4337	5214
	S.D.	2684	1454	2629	1774	1769	1964
	N	4	4	4	4	4	4
T helper cells	Mean	3167	1638	2555	2560	2254	2700
	S.D.	1115	806	1252	1227	684	796
	N	4	4	4	4	4	4
T cytotoxic cell	Mean	2778	1066	2223	2105	2052	2453
	S.D.	1868	826	1779	951	1228	1397
	N	4	4	4	4	4	4
B cells	Mean	2217	1948	2064	1994	2269	2289
	S.D.	1583	1576	1472	1240	1718	1511
	N	4	4	4	4	4	4
NK cells	Mean	803	266	673	671	721	782
	S.D.	455	174	387	412	378	432
	N	4	4	4	4	4	4
Monocyte	Mean	727	1028	934	860	628	605
	S.D.	413	467	242	356	292	338
	N	4	4	4	4	4	4

Group 4 (IV) - 125 mg/kg - Lymphocyte subsets and Monocytes (cells/μL of blood) - ***Males***

Sampling Time		D1(pre-treat)	D1(after 3 hrs)	D7	D14	D21	D28
T cells	Mean	3836	2848	4486	3899	4041	4016
	S.D.	1541	1304	1826	1601	2087	2841
	N	5	5	5	5	5	5
T helper cells	Mean	1983	1687	2363	2142	2055	1689
	S.D.	793	878	979	861	908	657
	N	5	5	5	5	5	5
T cytotoxic cell	Mean	1851	1159	2116	1754	1980	1370
	S.D.	970	452	1020	833	1351	391
	N	5	5	5	5	5	5
B cells	Mean	2482	2353	2567	2366	2611	2331
	S.D.	1499	1749	1732	1465	1436	1363
	N	5	5	5	5	5	5
NK cells	Mean	978	462	922	717	826	733
	S.D.	377	325	339	304	177	255
	N	5	5	5	5	5	5
Monocyte	Mean	715	858	641	706	506	786
	S.D.	353	527	204	268	442	447
	N	5	5	5	5	5	5

Group 4 (IV) - 125 mg/kg - Lymphocyte subsets and Monocytes (cells/μL of blood) - ***Males***

Sampling Time		W2 rec	W4 rec	W6 rec	W8 rec	W11 rec	W14 rec	W18 rec	W23 rec	W29 rec
T cells	Mean	3434	2693	6753	5915	5678	6377	6751	7519	5332
	S.D.	1350	1628	3516	1175	1909	1896	2583	2924	1126
	N	2	2	2	2	2	2	2	2	2
T helper cells	Mean	1564	1407	3095	2666	2545	2922	3131	3523	2788
	S.D.	576	646	1276	185	421	257	414	540	98
	N	2	2	2	2	2	2	2	2	2
T cytotoxic cell	Mean	1772	1264	3766	3267	3152	3431	3670	3972	2507
	S.D.	726	951	2393	989	1470	1640	2131	2384	975
	N	2	2	2	2	2	2	2	2	2
B cells	Mean	1875	1610	1995	1569	1784	1509	1881	1526	1231
	S.D.	455	78	270	245	109	64	294	260	281
	N	2	2	2	2	2	2	2	2	2
NK cells	Mean	653	380	691	739	608	598	698	623	622
	S.D.	366	249	69	366	173	148	135	127	322
	N	2	2	2	2	2	2	2	2	2
Monocyte	Mean	482	635	728	599	370	439	650	509	718
	S.D.	104	337	342	64	272	45	63	80	202
	N	2	2	2	2	2	2	2	2	2

Group 4 (IV) - 125 mg/kg - Lymphocyte subsets and Monocytes (cells/μL of blood) - ***Females***

Sampling Time		D1(pre-treat)	D1(after 3 hrs)	D7	D14	D21	D28
T cells	Mean	5384	2550	4398	3922	3496	4260
	S.D.	2679	1846	2109	1927	2194	2178
	N	5	5	5	5	5	5
T helper cells	Mean	2875	1529	2470	2135	1907	2334
	S.D.	1207	1031	1089	905	1095	1081
	N	5	5	5	5	5	5
T cytotoxic cell	Mean	2515	1021	1955	1784	1583	1925
	S.D.	1566	819	1074	1079	1174	1203
	N	5	5	5	5	5	5
B cells	Mean	1165	1033	1013	983	1014	1251
	S.D.	656	838	722	555	659	783
	N	5	5	5	5	5	5
NK cells	Mean	892	273	694	692	553	965
	S.D.	346	147	448	354	248	543
	N	5	5	5	5	5	5
Monocyte	Mean	781	840	649	407	646	609
	S.D.	39	321	292	167	340	208
	N	5	5	5	5	5	5

Group 4 (IV) - 125 mg/kg - Lymphocyte subsets and Monocytes (cells/μL of blood) - ***Females***

Sampling Time		W2 rec	W4 rec	W6 rec	W8 rec	W11 rec	W14 rec	W18 rec	W23 rec	W29 rec
T cells	Mean	3110	3554	4754	4143	4463	4474	4457	4591	4351
	S.D.	2195	2720	3424	2701	3055	3454	2883	3177	2203
	N	2	2	2	2	2	2	2	2	2
T helper cells	Mean	1775	1937	2419	2149	2332	2324	2338	2374	2416
	S.D.	1406	1526	2015	1604	1741	1959	1590	1755	1162
	N	2	2	2	2	2	2	2	2	2
T cytotoxic cell	Mean	1336	1628	2341	2016	2112	2139	2186	2168	1858
	S.D.	846	1208	1415	1129	1315	1505	1199	1346	1129
	N	2	2	2	2	2	2	2	2	2
B cells	Mean	1083	1496	1225	1243	980	1003	1018	1170	1245
	S.D.	824	1258	936	949	725	812	690	993	975
	N	2	2	2	2	2	2	2	2	2
NK cells	Mean	754	765	802	675	708	731	694	763	691
	S.D.	515	409	166	332	171	428	372	376	241
	N	2	2	2	2	2	2	2	2	2
Monocyte	Mean	692	736	684	975	793	831	710	589	936
	S.D.	158	487	86	149	139	151	203	155	0
	N	2	2	2	2	2	2	2	2	2

Group 5 (SC) - 75 mg/kg - Lymphocyte subsets and Monocytes (cells/µL of blood) - ***Males***

Sampling Time		D1(pre-treat)	D1(after 6 hrs)	D7	D14	D21	D28
T cells	Mean	3519	2596	3162	3274	2595	3193
	S.D.	1250	745	777	880	1616	984
	N	5	5	5	5	5	5
T helper cells	Mean	2065	1543	1759	1888	1525	1732
	S.D.	1290	734	771	983	1112	974
	N	5	5	5	5	5	5
T cytotoxic cell	Mean	1464	1046	1397	1391	1078	1466
	S.D.	585	125	678	493	614	660
	N	5	5	5	5	5	5
B cells	Mean	1156	1712	1083	1160	1200	1094
	S.D.	494	417	657	588	710	631
	N	5	5	5	5	5	5
NK cells	Mean	892	314	715	555	678	702
	S.D.	685	255	547	408	647	413
	N	5	5	5	5	5	5
Monocyte	Mean	334	768	487	454	346	491
	S.D.	238	301	237	181	99	174
	N	5	5	5	5	5	5

Group 5 (SC) - 75 mg/kg - Lymphocyte subsets and Monocytes (cells/µL of blood) - ***Males***

Sampling Time		W2 rec	W4 rec	W6 rec	W8 rec	W11 rec	W14 rec	W18 rec	W23 rec	W29 rec
T cells	Mean	3171	2875	4514	3909	3721	4323	4767	6175	4611
	S.D.	1276	540	1223	903	506	742	907	512	2057
	N	2	2	2	2	2	2	2	2	2
T helper cells	Mean	1818	1527	2513	2012	1861	2195	2632	3080	2734
	S.D.	1800	1288	1990	1505	1440	1639	1788	2151	2384
	N	2	2	2	2	2	2	2	2	2
T cytotoxic cell	Mean	1350	1366	2022	1916	1860	2119	2136	3083	1900
	S.D.	540	756	754	603	928	898	962	1665	264
	N	2	2	2	2	2	2	2	2	2
B cells	Mean	1842	1665	1568	1201	1138	898	1226	849	857
	S.D.	255	263	158	417	168	237	237	102	192
	N	2	2	2	2	2	2	2	2	2
NK cells	Mean	537	640	532	539	505	399	450	799	547
	S.D.	264	140	91	96	146	185	153	213	115
	N	2	2	2	2	2	2	2	2	2
Monocyte	Mean	464	568	575	622	393	470	574	315	198
	S.D.	153	52	40	216	158	4	9	413	270
	N	2	2	2	2	2	2	2	2	2

Group 5 (SC) - 75 mg/kg - Lymphocyte subsets and Monocytes (cells/µL of blood) - ***Females***

Sampling Time		D1(pre-treat)	D1(after 6 hrs)	D7	D14	D21	D28
T cells	Mean	6574	5966	6638	5318	4423	5280
	S.D.	2352	2719	2707	1156	883	875
	N	5	5	5	5	5	5
T helper cells	Mean	3797	3467	3944	3130	2656	3072
	S.D.	1073	1409	1428	606	591	551
	N	5	5	5	5	5	5
T cytotoxic cell	Mean	2787	2495	2692	2171	1751	2189
	S.D.	1315	1324	1287	548	324	359
	N	5	5	5	5	5	5
B cells	Mean	1340	2297	1706	1354	1348	1474
	S.D.	557	726	1088	476	532	730
	N	5	5	5	5	5	5
NK cells	Mean	1136	753	1027	824	800	950
	S.D.	650	454	527	367	375	418
	N	5	5	5	5	5	5
Monocyte	Mean	1309	966	663	748	451	832
	S.D.	1160	964	174	485	254	347
	N	5	5	5	5	5	5

Group 5 (SC) - 75 mg/kg - Lymphocyte subsets and Monocytes (cells/µL of blood) - ***Females***

Sampling Time		W2 rec	W4 rec	W6 rec	W8 rec	W11 rec	W14 rec	W18 rec	W23 rec	W29 rec
T cells	Mean	5780	4945	7676	8465	8615	8354	10388	7699	7954
	S.D.	2406	1732	2541	3164	3827	2581	2843	746	1316
	N	2	2	2	2	2	2	2	2	2
T helper cells	Mean	3342	2929	3878	4580	4557	4574	5501	4236	4666
	S.D.	1501	1307	1365	1690	1894	1245	1230	238	998
	N	2	2	2	2	2	2	2	2	2
T cytotoxic cell	Mean	2454	2003	3805	3927	4042	3717	4870	3425	3297
	S.D.	902	432	1223	1503	1990	1447	1558	629	406
	N	2	2	2	2	2	2	2	2	2
B cells	Mean	1652	1955	2120	2607	2576	2205	2459	2180	2383
	S.D.	1168	1223	1456	1864	1962	1462	1201	1143	1168
	N	2	2	2	2	2	2	2	2	2
NK cells	Mean	1123	1427	1422	1373	1342	1355	1266	1186	875
	S.D.	19	248	196	23	66	190	17	74	51
	N	2	2	2	2	2	2	2	2	2
Monocyte	Mean	623	632	777	467	722	521	1045	785	512
	S.D.	9	50	499	421	383	11	120	19	48
	N	2	2	2	2	2	2	2	2	2

Table 10: Hematological parameters - (Mean ± S.D.)

Group 1 (IV) - 0 mg/kg - Hematological parameters (cells/μL of blood) – ***Males***

Sampling Time		D1(pre-treat)	D1(after 3 hrs)	D7	D14	D21	D28
Lymphocytes	Mean	7562.5	4797.5	7072.5	7675.0	6995.0	6917.5
	S.D.	1282.0	1416.7	1382.9	3105.6	3551.8	1596.9
	N	4	4	4	4	4	4
Neutrophils	Mean	8081.3	15025.0	4610.0	5527.5	5727.5	6697.5
	S.D.	2753.4	5336.9	2064.4	3256.9	3223.1	4076.4
	N	4	4	4	4	4	4
WBC	Mean	16425.0	20725.0	12437.5	13925.0	13400.0	14225.0
	S.D.	2093.4	5736.7	2150.0	2035.3	2353.7	3214.9
	N	4	4	4	4	4	4

Group 1 (i.v.) - 0 mg/kg - Hematological parameters (cells/μL of blood) - ***Females***

Sampling Time		D1(pre-treat)	D1(after 3 hrs)	D7	D14	D21	D28
Lymphocytes	Mean	7527.5	5950.0	5610.0	6297.5	5872.5	6672.5
	S.D.	2122.2	2113.4	2210.1	1540.8	1462.2	1886.4
	N	4	4	4	4	4	4
Neutrophils	Mean	4485.0	12892.5	4847.5	3925.0	3905.0	5540.0
	S.D.	974.0	4602.5	1475.1	652.8	1242.4	5766.4
	N	4	4	4	4	4	4
WBC	Mean	13250.0	19675.0	11227.5	10920.0	10330.0	13475.0
	S.D.	2074.4	3082.6	2586.0	1492.7	1693.7	5385.4
	N	4	4	4	4	4	4

Group 2 (IV) - 5 mg/kg - Hematological parameters (cells/μL of blood) - ***Males***

Sampling Time		D1(pre-treat)	D1(after 3 hrs)	D7	D14	D21	D28
Lymphocytes	Mean	6290.0	4752.5	6022.5	5535.0	6095.0	6525.0
	S.D.	1500.6	2309.3	1546.4	1705.9	2049.2	892.0
	N	4	4	4	4	4	4
Neutrophils	Mean	4862.5	12750.0	5832.5	8440.0	5087.5	6627.5
	S.D.	3128.3	6281.6	2709.1	4878.2	4209.7	3829.1
	N	4	4	4	4	4	4
WBC	Mean	11665.0	18250.0	12535.0	14875.0	11667.5	14000.0
	S.D.	2737.1	4636.4	2297.2	4006.1	4190.2	2942.8
	N	4	4	4	4	4	4

Group 2 (IV) - 5 mg/kg - Hematological parameters (cells/μL of blood) - ***Females***

Sampling Time		D1(pre-treat)	D1(after 3 hrs)	D7	D14	D21	D28
Lymphocytes	Mean	9230.0	5180.0	6440.0	6700.0	5712.5	6435.0
	S.D.	3808.3	704.1	2447.0	1849.2	2079.0	3850.4
	N	3	4	4	4	4	4
Neutrophils	Mean	4750.0	11812.5	6687.5	4837.5	7145.0	5135.0
	S.D.	1669.1	5562.4	2243.1	2299.2	4591.6	2389.4
	N	3	4	4	4	4	4
WBC	Mean	15066.7	18175.0	14450.0	12472.5	13547.5	13097.5
	S.D.	5564.5	6260.1	4221.0	2518.8	6414.1	5293.9
	N	3	4	4	4	4	4

Group 3 (IV) - 25 mg/kg - Hematological parameters (cells/µL of blood) - ***Males***

Sampling Time		D1(pre-treat)	D1(after 3 hrs)	D7	D14	D21	D28
Lymphocytes	Mean	4700.0	4057.5	4365.0	4215.0	4397.5	4712.5
	S.D.	920.4	900.6	670.8	540.7	297.4	1324.1
	N	4	4	4	4	4	4
Neutrophils	Mean	9605.0	14475.0	5992.5	6185.0	3505.0	8450.0
	S.D.	4985.7	3796.8	2456.8	3996.0	1682.4	5087.4
	N	4	4	4	4	4	4
WBC	Mean	16912.5	19350.0	10837.5	10945.0	8255.0	13852.5
	S.D.	5343.6	4805.9	2914.7	3684.6	2009.0	5736.7
	N	4	4	4	4	4	4

Group 3 (IV) - 25 mg/kg - Hematological parameters (cells/µL of blood) - ***Females***

Sampling Time		D1(pre-treat)	D1(after 3 hrs)	D7	D14	D21	D28
Lymphocytes	Mean	9047.5	4965.0	7555.0	7445.0	7542.5	8315.0
	S.D.	4034.2	2991.2	4065.4	3049.2	3096.4	3113.7
	N	4	4	4	4	4	4
Neutrophils	Mean	4552.5	13150.0	4480.0	4607.5	4762.5	2625.0
	S.D.	1245.7	2200.8	596.2	2723.3	2904.7	1312.8
	N	4	4	4	4	4	4
WBC	Mean	14485.0	19225.0	12877.5	12860.0	13087.5	11710.0
	S.D.	5502.6	4377.5	4612.0	5779.4	4856.9	3659.0
	N	4	4	4	4	4	4

Group 4 (IV) - 125 mg/kg - Hematological parameters (cells/µL of blood)

Lymphocytes

Sampling time		D1(pre-treat)	D1(after 3 hrs)	D7	D14	D21	D28
Males	Mean	7194.0	5696.0	7898.0	6904.0	7482.0	6210.0
	S.D.	2794.4	3088.5	3580.6	2943.3	3253.3	2104.9
	N	5	5	5	5	5	5
Females	Mean	7520.0	3928.0	6212.0	5754.0	5200.0	6476.0
	S.D.	3162.5	2735.3	2781.2	2404.5	2703.2	2852.9
	N	5	5	5	5	5	5

Sampling time		W2 rec	W4 rec	W6 rec	W8 rec	W11 rec	W14 rec	W18 rec	W23 rec	W29 rec
Males	Mean	6135.0	4765.0	9830.0	8465.0	8615.0	8820.0	9830.0	10190.0	7510.0
	S.D.	473.8	1831.4	4058.8	530.3	2100.1	1810.2	2786.0	2984.0	1060.7
	N	2	2	2	2	2	2	2	2	2
Females	Mean	4940.0	5925.0	6980.0	6250.0	6625.0	6470.0	6405.0	6815.0	6560.0
	S.D.	3380.0	4377.0	4553.8	3988.1	4065.9	4780.0	3867.9	4645.7	3521.4
	N	2	2	2	2	2	2	2	2	2

Neutrophils

Sampling time		D1(pre-treat)	D1(after 3 hrs)	D7	D14	D21	D28
Males	Mean	7362.0	14120.0	4190.0	5272.0	2384.0	7065.6
	S.D.	4136.1	3428.8	1007.4	1948.6	604.2	4048.7
	N	5	5	5	5	5	5
Females	Mean	5316.6	12524.0	5740.0	3582.0	4230.0	3458.0
	S.D.	5763.4	4551.5	1799.9	1849.6	2142.3	2432.7
	N	5	5	5	5	5	5

Sampling time		W2 rec	W4 rec	W6 rec	W8 rec	W11 rec	W14 rec	W18 rec	W23 rec	W29 rec
Males	Mean	2560.0	6270.0	4205.0	2935.0	2730.0	2055.0	3690.0	3810.0	5545.0
	S.D.	1088.9	2998.1	1506.1	502.0	240.4	346.5	1527.4	749.5	601.0
	N	2	2	2	2	2	2	2	2	2
Females	Mean	1940.0	3245.0	3150.0	4050.0	3732.0	3885.0	3190.0	2260.0	6340.0
	S.D.	523.3	2284.0	1979.9	919.2	3914.5	700.0	2121.3	297.0	3578.0
	N	2	2	2	2	2	2	2	2	2

WBC

Sampling time		D1(pre-treat)	D1(after 3 hrs)	D7	D14	D21	D28
Males	Mean	15370.0	20680.0	12888.0	13040.0	10522.0	14260.0
	S.D.	6315.8	3476.6	3891.2	2133.8	3194.6	3617.0
	N	5	5	5	5	5	5
Females	Mean	13784.0	17360.0	12780.0	9850.0	8242.0	10788.0
	S.D.	5995.7	3592.1	1351.7	2617.3	4254.6	3858.1
	N	5	5	5	5	5	5

Sampling time		W2 rec	W4 rec	W6 rec	W8 rec	W11 rec	W14 rec	W18 rec	W23 rec	W29 rec
Males	Mean	9280.0	11750.0	15000.0	12200.0	11900.0	11550.0	14400.0	14700.0	14450.0
	S.D.	495.0	777.8	5798.3	989.9	2687.0	1343.5	1272.8	3818.4	1484.9
	N	2	2	2	2	2	2	2	2	2
Females	Mean	7700.0	10110.0	11100.0	11495.0	11505.0	11495.0	10860.0	10155.0	13700.0
	S.D.	4101.2	1682.9	6646.8	5239.7	8478.2	4532.6	6279.1	5579.1	424.3
	N	2	2	2	2	2	2	2	2	2

Group 5 (SC) - 75 mg/kg - Hematological parameters (cells/μL of blood)

Lymphocytes

Sampling time		D1(pre-treat)	D1(after 6 hrs)	D7	D14	D21	D28
Males	Mean	5484.0	4722.0	4942.0	5002.0	5030.0	4486.0
	S.D.	1967.2	1106.0	1413.2	1085.5	1532.6	2017.0
	N	5	5	5	5	5	5
Females	Mean	9080.0	9044.0	9368.0	7590.0	6748.0	7636.0
	S.D.	2953.4	3430.4	3787.0	1598.6	1351.0	1699.7
	N	5	5	5	5	5	5

Sampling time		W2 rec	W4 rec	W6 rec	W8 rec	W11 rec	W14 rec	W18 rec	W23 rec	W29 rec
Males	Mean	5585.0	5175.0	6730.0	5725.0	5625.0	5900.0	6810.0	8220.0	6295.0
	S.D.	770.7	459.6	1173.8	601.0	544.5	806.1	806.1	565.7	2100.1
	N	2	2	2	2	2	2	2	2	2
Females	Mean	8605.0	8350.0	11350.0	12820.0	13045.0	12285.0	14550.0	11400.0	11560.0
	S.D.	3528.5	2757.7	3747.7	5204.3	6017.5	4122.4	4030.5	1838.5	2319.3
	N	2	2	2	2	2	2	2	2	2

Neutrophils

Sampling time		D1(pre-treat)	D1(after 6 hrs)	D7	D14	D21	D28
Males	Mean	4768.0	6736.0	5312.0	4224.0	3938.0	6680.0
	S.D.	2913.3	1715.0	1606.0	2243.3	1820.4	3761.5
	N	5	5	5	5	5	5
Females	Mean	5202.0	7394.0	5002.0	5594.0	4042.0	3986.0
	S.D.	1162.7	567.8	2779.0	2470.7	1777.3	1951.9
	N	5	5	5	5	5	5

Sampling time		W2 rec	W4 rec	W6 rec	W8 rec	W11 rec	W14 rec	W18 rec	W23 rec	W29 rec
Males	Mean	3735.0	3800.0	3140.0	3200.0	3940.0	2290.0	2735.0	2650.0	2345.0
	S.D.	1534.4	2446.6	735.4	989.9	2644.6	1202.1	1718.3	1711.2	968.7
	N	2	2	2	2	2	2	2	2	2
Females	Mean	4815.0	4515.0	3520.0	4470.0	3075.0	3610.0	5145.0	2400.0	3955.0
	S.D.	2481.9	869.7	1032.4	876.8	1605.1	1583.9	1520.3	551.5	926.3
	N	2	2	2	2	2	2	2	2	2

WBC

Sampling time		D1(pre-treat)	D1(after 6 hrs)	D7	D14	D21	D28
Males	Mean	10624.0	12360.0	10846.0	9828.0	9468.0	11576.0
	S.D.	3261.3	1569.4	2052.1	1411.9	709.8	2656.3
	N	5	5	5	5	5	5
Females	Mean	15840.0	17560.0	15260.0	14108.0	11458.0	12720.0
	S.D.	5122.8	4733.2	6325.6	4306.9	2309.2	3766.6
	N	5	5	5	5	5	5

Sampling time		W2 rec	W4 rec	W6 rec	W8 rec	W11 rec	W14 rec	W18 rec	W23 rec	W29 rec
Males	Mean	9885.0	9635.0	10600.0	9755.0	10070.0	8780.0	10255.0	11300.0	8885.0
	S.D.	586.9	1930.4	424.3	120.2	2305.2	367.7	912.2	1555.6	883.9
	N	2	2	2	2	2	2	2	2	2
Females	Mean	14385.0	13750.0	16100.0	18200.0	17100.0	16700.0	21500.0	14950.0	16300.0
	S.D.	6385.2	3889.1	5798.3	6929.6	8202.4	5939.7	2404.2	2474.9	1555.6
	N	2	2	2	2	2	2	2	2	2

5.6 OTHER BIOMARKERS

5.6.1 Natural killer (NK) cell activity

Mean and SD values of NK cell activity as obtained at the various time of analysis are graphically reported in ***Figure 47***.

No treatment-related effects were appreciable among the dosed groups.
The lower values observed in animals dosed at 25 mg/kg at week 5 of treatment was considered incidental, and therefore without any biological relevance.

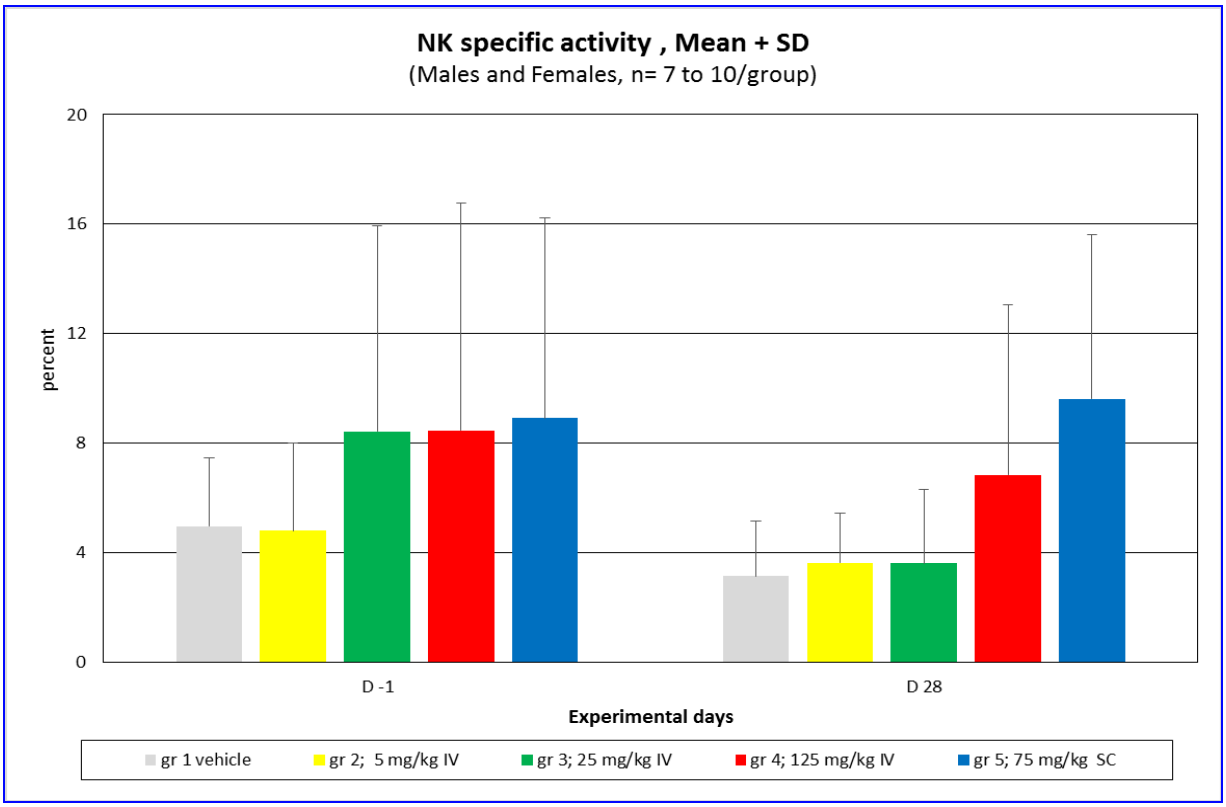


Fig. 47: *Natural Killer cell activity – Males & Females (monkeys)*

5.6.2 CRS MARKERS (TNFα, IL-6, IFNγ, IL-1β, IL-2, IL-4, IL-10, IL-8, MCP-1)

IL-1β, IL-2 and IFN-γ (***Figure 48***) as well as TNF-α, IL-4 and IL-10 (***Figure 49***) did not show any significant change over time.

Nor was any effect seen with IL-8. MCP-1 showed a time-effect but this was not statistically significant (***Figure 50***).

The modulation was similar both in the control group and in the treated groups.

Of note, IL-6 level showed a rise at 2h and 6h after the administration of the two higher i.v.doses of Anti-CD25.
This was observed both after the 1st and the 5th administration with the female animals showing a slightly higher response (Group 4) (***Figure 51***).
The increase in IL6 was also seen once time as clearly reported in (***Figure 52***).
This effect indicates the need to evaluate further such possibility at clinically relevant doses before any exposure.

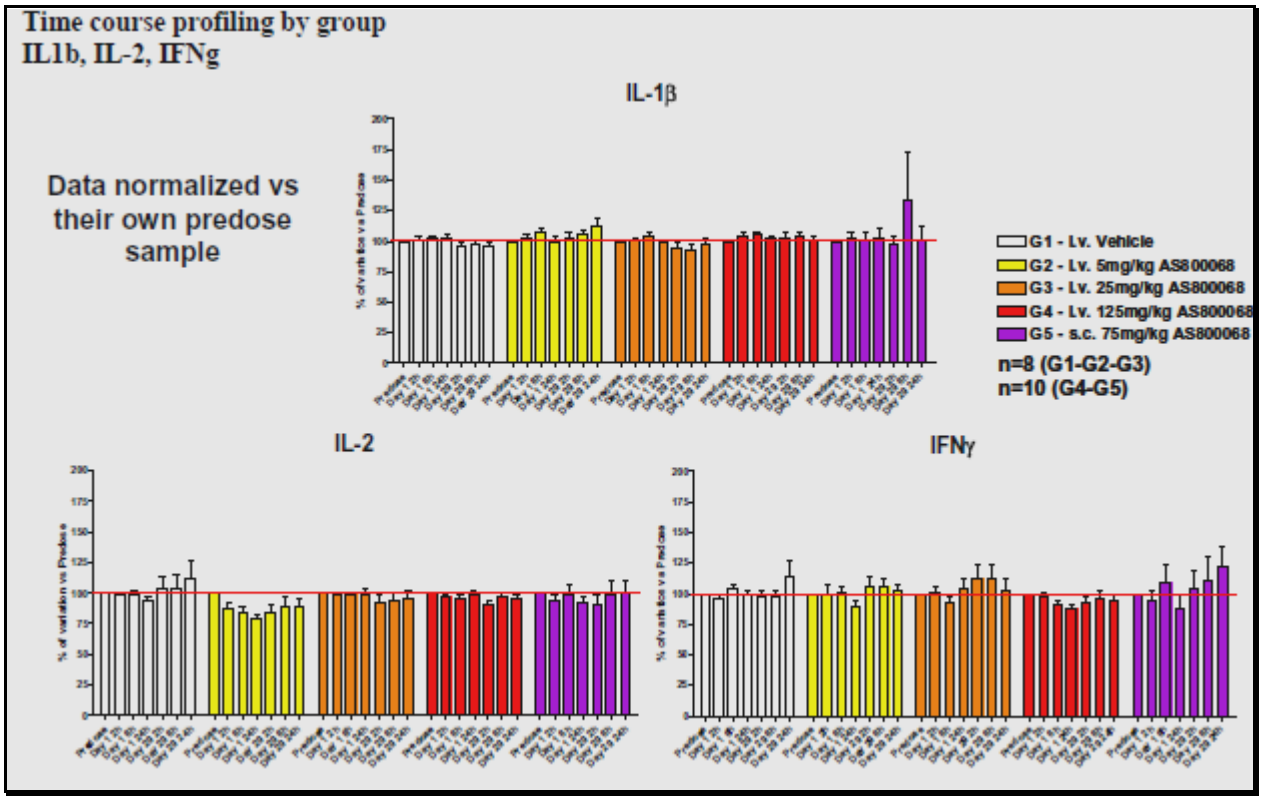


Fig. 48: *IL-1β, IL-2 and IFN-γ effect over time - control group and all treated groups (monkeys)*

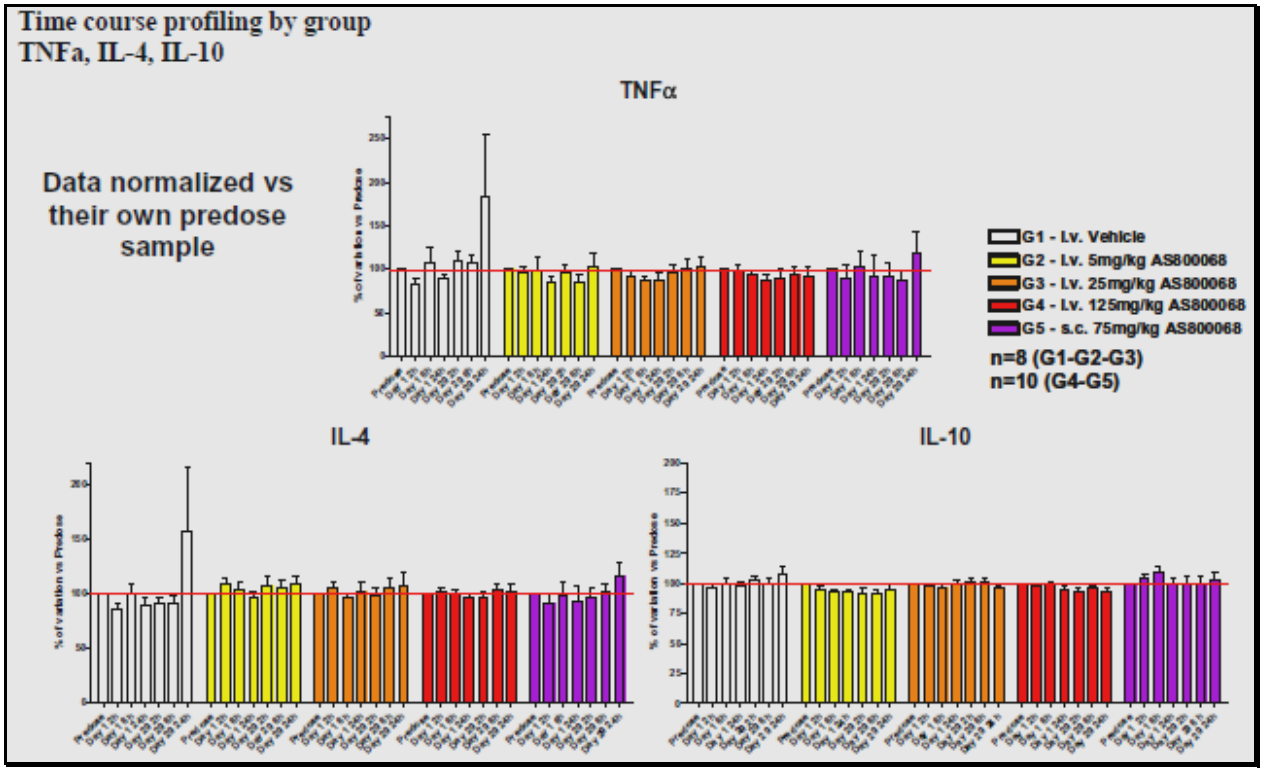


Fig. 48: *TNF-α, IL-4 and IL-10 effect over time - control group and all treated groups (monkeys)*

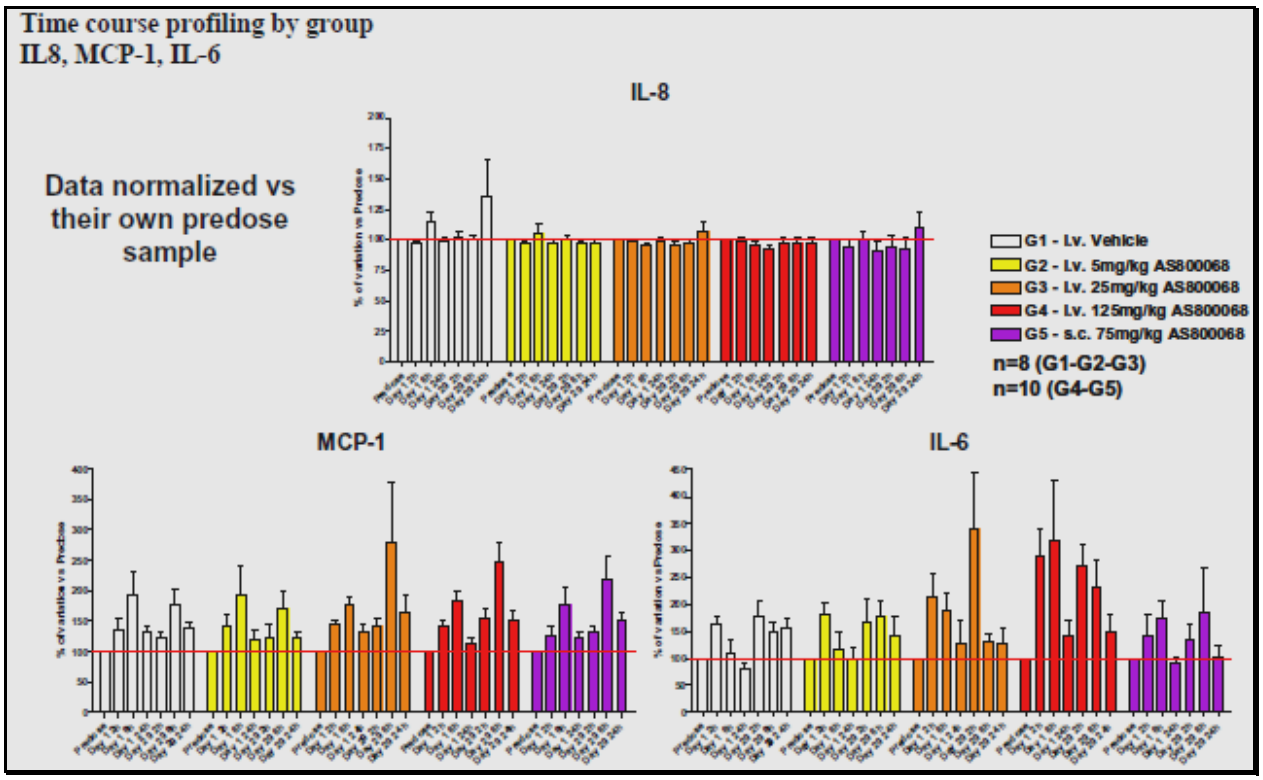


Fig. 49: *IL-8, MCP-1, lL-6 effect over time - control group and all treated groups (monkeys)*

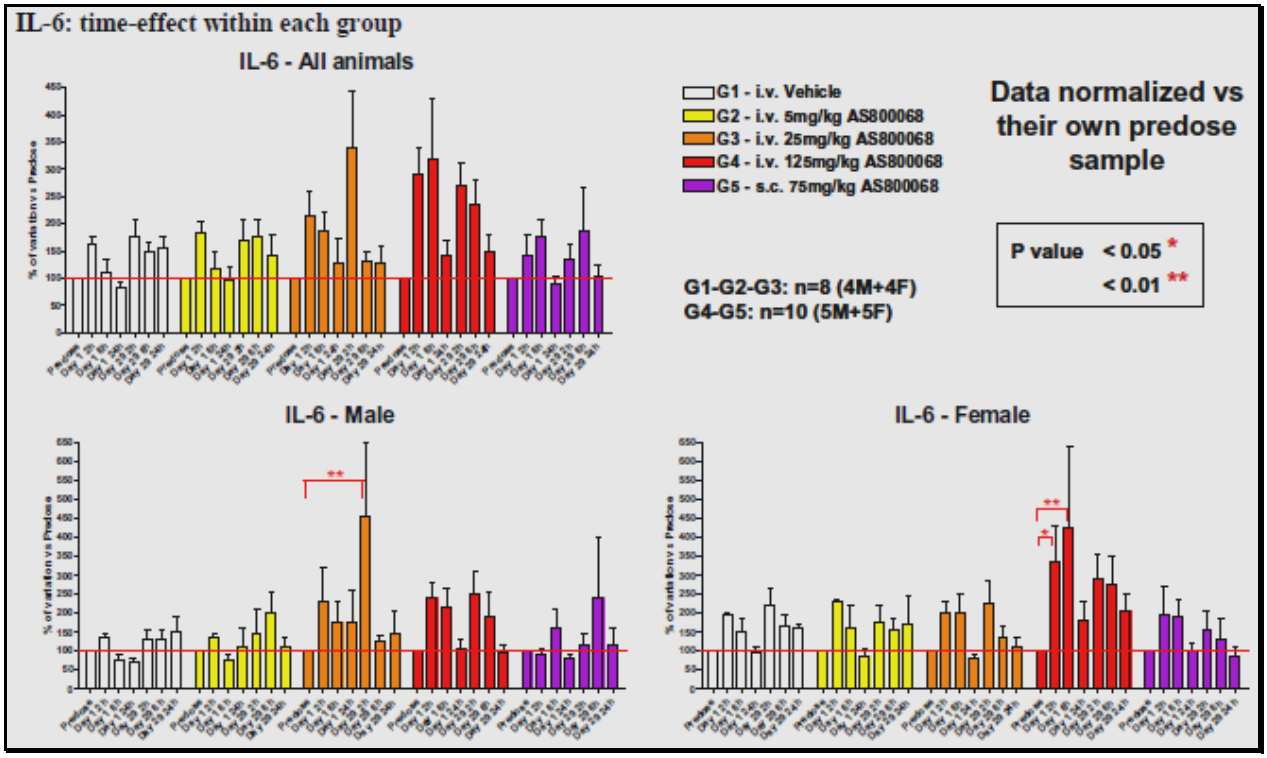


***Fig. 50**: lL-6 time- effect within each group - Males & Females (monkeys)*

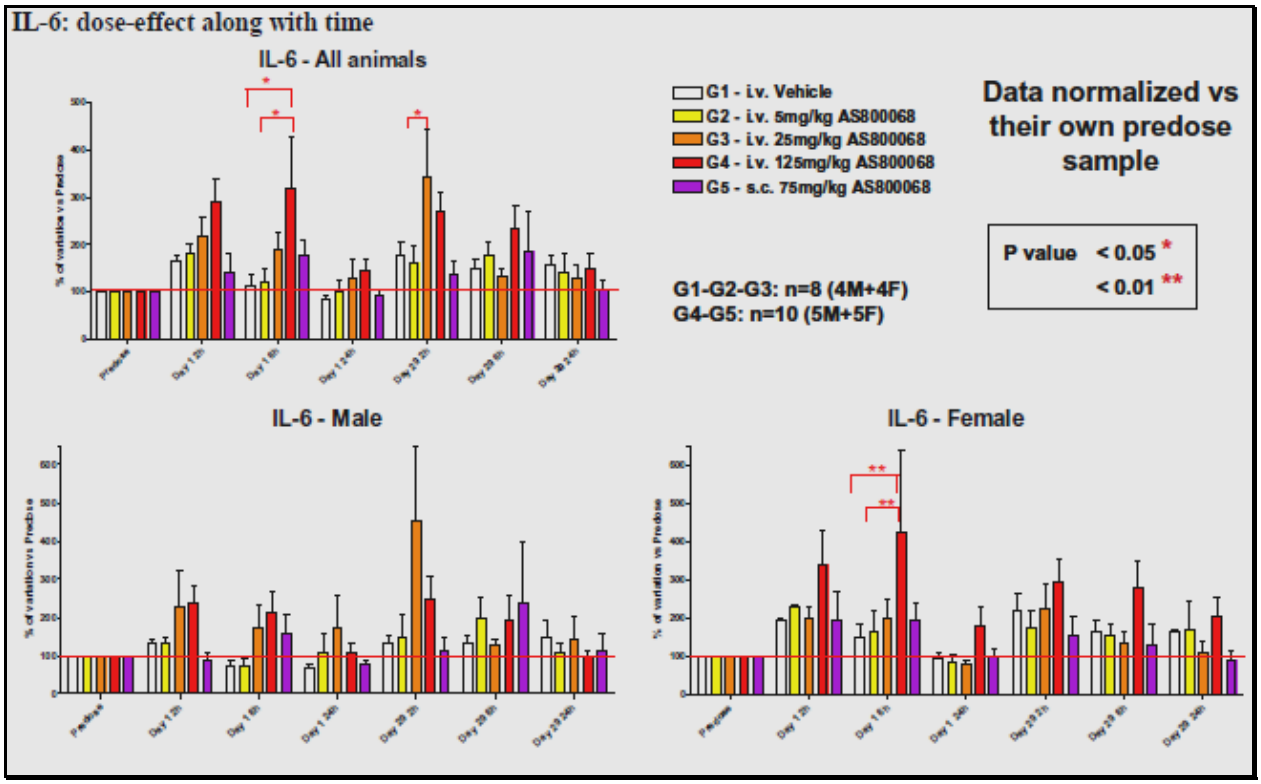


***Fig. 51:** lL-6 dose- effect over time within each group - Males & Females (monkeys)*

5.7 ANTIBODY DETERMINATION

The method used for the analysis showed that a concentration of 500 ng/mL of Anti-CD25 in the sample interfere with the assay.
Only male animals of all the treated groups (from group 2 to group 5) were found positives for the presence of binding antibodies to Anti-CD25 (***Table 11***).
Antibodies were revealed starting from week 6 of the recovery period with a mean titer of >100.
They were detected till the last week of recovery. The positivity remained until the last week of the recovery.

Table 11: Antibodies determination – Individual values

Group 1 (0 mg/Kg - IV)	time	Period 2 An. 0406025M rec	Period 3 An.0309164F rec
	Pre-dose	Neg.	Neg.
recovery	w1	Neg.	Neg.
recovery	w4	Neg.	Neg.
recovery	w6	Neg.	Neg.
recovery	w8	Neg.	Neg.
recovery	w27M - w26F	Neg.	Neg.
recovery	w32	Neg.	Neg.

Group 2 (5 mg/Kg - IV)	time	Period 2 An. 0401043M rec	Period 3 An.0401208F rec
	Pre-dose	Neg.	Neg.
recovery	w1	Neg.	Neg.
recovery	w4	Neg.	Neg.
recovery	w6	Neg.	Neg.
recovery	w8	Neg.	Neg.
recovery	w27M - w26F	Pos. >1000	Neg.
recovery	w32	Pos. >100	Neg.

Group 3 (25 mg/Kg -IV)	time	Period 2 Result	An. 0402027M rec Titer	Period 3 Result	An.0312490F rec Titer
recovery	Pre-dose	Neg.	===	Neg.	===
recovery	W1	Neg.	===	Neg.	===
recovery	w4	Neg.	===	Neg.	===
recovery	w6	Pos.	>>1000	Neg.	===
recovery	w8	Pos.	>>1000	Neg.	===
recovery	w27M - w26F	Pos.	>>1000	Neg.	===
recovery	w32	Pos.	>>1000	Neg.	===

Group 4 (125 mg/Kg - IV)	time	Period 2 An. 0310411M rec	An.0404015M rec	Period 3 An.0312210F rec	An.0402134F rec
	Pre-dose	Neg.	Neg.	Neg.	Neg.
recovery	w1	Neg.	Neg.	Neg.	Neg.
recovery	w4	Neg.	Neg.	Neg.	Neg.
recovery	w6	Neg.	Neg.	Neg.	Neg.
recovery	w8	Neg.	Neg.	Neg.	Neg.
recovery	w27M - w26F	Pos.>100	Neg.	Neg.	Neg.
recovery	w32	Neg.	Pos >100	Neg.	Neg.

Group 5 (75 mg/Kg - SC)	time	Period 2 An. 0312003M rec	An.0401037M rec	Period 3 An.0307140F rec	An.0401222F rec
	Pre-dose	Neg.	Neg.	Neg.	Neg.
recovery	w1	Neg.	Neg.	Neg.	Neg.
recovery	w4	Neg.	Neg.	Neg.	Neg.
recovery	w6	Neg.	Neg.	Neg.	Neg.
recovery	w8	Neg.	Neg.	Neg.	Neg.
recovery	w27M - w26F	Pos >1000	Pos.>1000	Neg.	Neg.
recovery	w32	Pos. >100	Pos. >1000	Neg.	Neg.

5.8 FUNCTIONAL TEST: T-cell Proliferation assay and T_{reg} cells number evaluation

The functional tests were set to evaluate the anti-proliferative and immunomodulatory effect of *Anti-CD25 mAb*.

T cell proliferation assay and *FoxP3 evaluation assay* were performed at the following time point: pre-dose, 24h after the last administration and at 3 recovery time-points (8, 18, 25 weeks after the last treatment).

T cell proliferation assay

As shown in ***Figure53***, 24 h after the last administration of 5 mg/kg IV, *Anti-CD25 mAb* did not affect the proliferation rate of anti-CD3, LPS, PHA and IL-2 stimulated cells, except a slight decrease ($p<0.05$, Student's t test vs predose) in the proliferation index of cells stimulated with IL-2 and LPS respectively in the group 3 and group 5 24h after the last treatment.

The recovery periods restored the initial values of proliferation.

The evaluation of T cell proliferation has been performed after each time point (predose and 24h after the last treatment), immediately after the bleeding, without any stimulation.

Anti-CD25 mAb administration for 5 weeks in a tox study IV and SC followed by 25 weeks recovery period in monkey demonstrated no effect on ex vivo T cell proliferation induced by anti-CD3 (0.1-1 µg/ml), IL-2 and LPS.

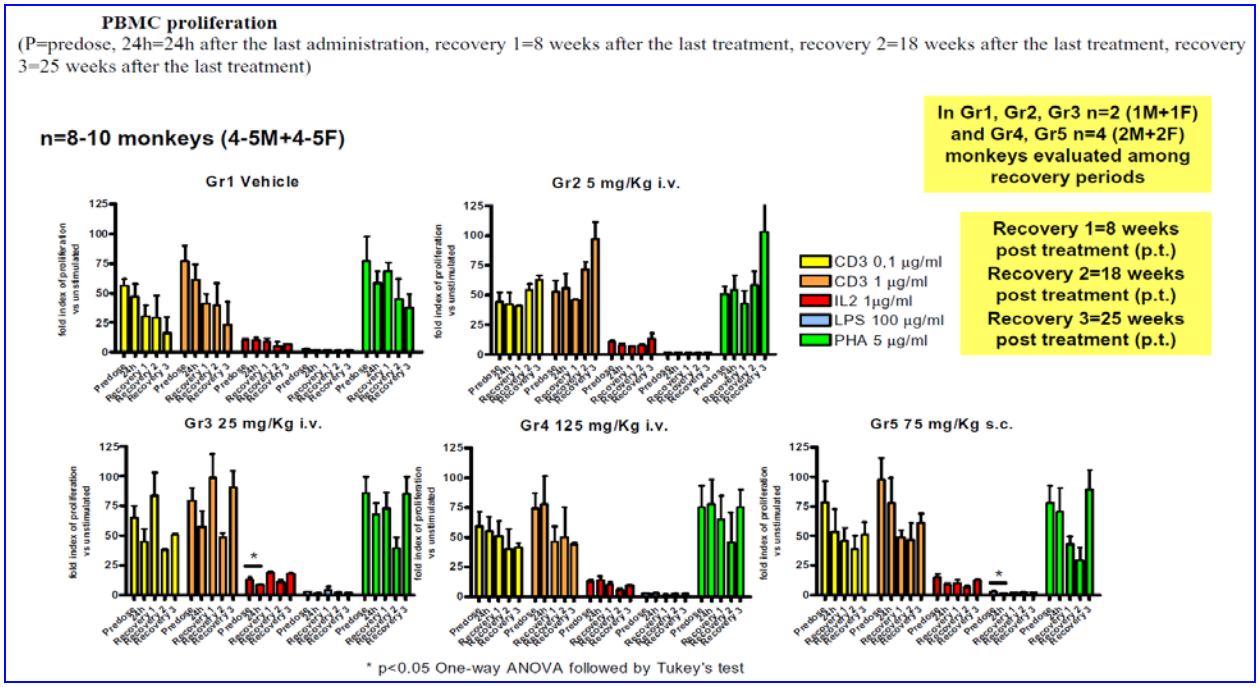


Fig. 52: *PBMCs proliferation over time - Males & Females (monkeys)*

FoxP3 evaluation assay:

Anti-CD25 mAb administration significantly decreased T regulatory cell percentage (CD4+CD25+FoxP3+).
In ***Figure 54***, representative dot-plots of two monkeys from group 1 (vehicle) and group 4 (125 mg/kg IV) are shown.
PBMCs have been stained for the surface markers CD4 and CD25, and subsequently intracellularly for FoxP3. After gating CD4+ cells, CD25+FoxP3+ cells percentages have been determined.
As shown in ***Figure 55***, T regulatory cell percentage was significantly decreased 24 h after *Anti-CD25 mAb* administration at 125 mg/kg IV (group 4) and 75 mg/kg SC (group 5) ($p<0.05$ Student's t test vs predose).
In ***Figure 56***, T_{reg} cells (CD4+CD25+FoxP3+, in light blue) are shown together with CD4+CD25+ (yellow), CD4+FoxP3+ (red) and CD25+FoxP3+ cell (orange) percentages.
The group 4 (125 mg/kg IV) and group 5 (75 mg/kg IV) demonstrated the same decrease of CD25+FoxP3+ cells 24 after the last treatment vs the pre-dose time point.
This evidence strengthen the detected effect of *Anti-CD25 mAb* on the T_{reg} cells frequency.
On the other hand, in the group 5 (75 mg/kg SC) a significant decrease of CD4+CD25+ cells has been observed. This could be associated to the CD25 receptor down-regulation that occurs during *Anti-CD25 mAb* administration. Notably in the group 5, the recovery periods were not able to restore the initial T_{reg} cell rate and, 25 week after treatment T_{reg} cell percentage is still lower than the predose value.
In conclusion, *Anti-CD25 mAb* significantly decreased the percentage of T regulatory cells 24h after the last administration of 125 mg/kg IV and 75 mg/kg SC.
This effect seems to correlate with the CD25 receptor down-regulation that occurs after administration of *Anti-CD25 mAb* for the 125 mg/kg IV group.
On the other hand the decrease of T_{reg} cells in the group treated with 75 mg/kg SC seems to be closely linked to the decrease of the FoxP3 transcription factor.

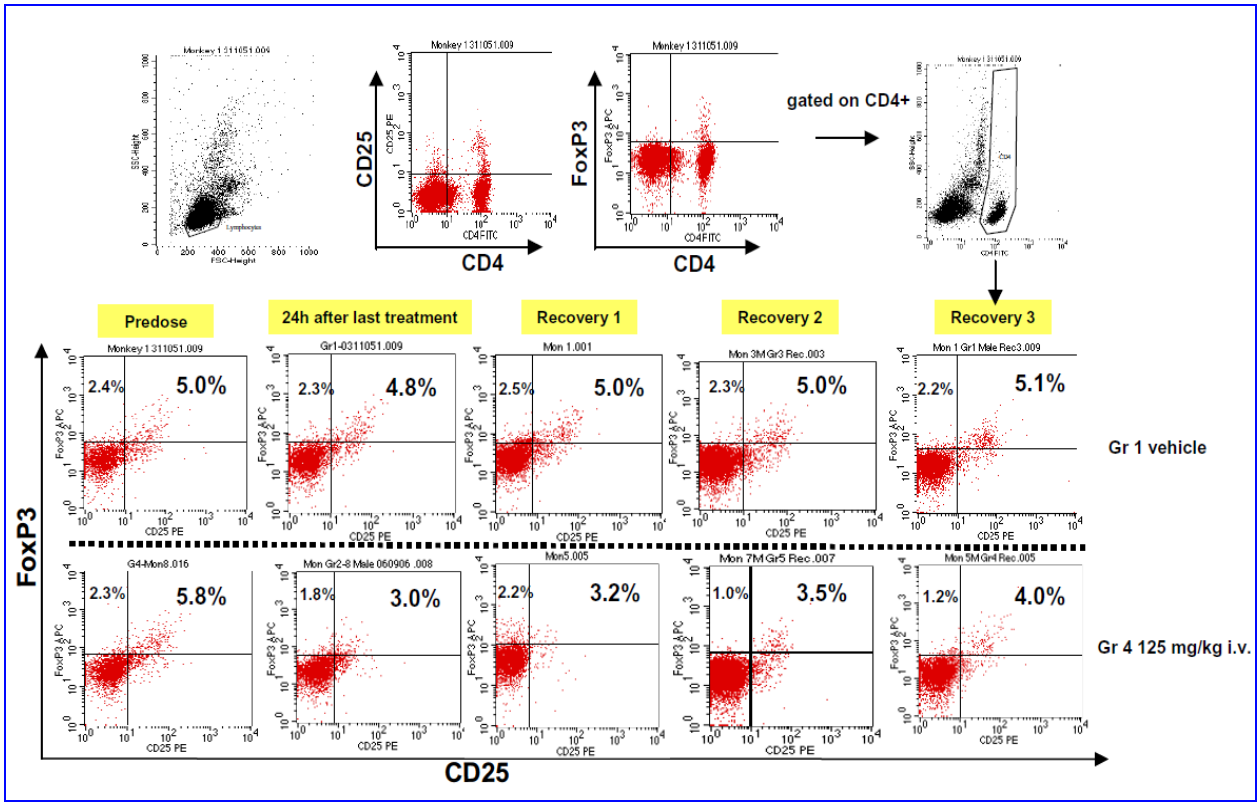


Fig. 53:*FoxP3 staining - dot plots - Males & Females (monkeys)*

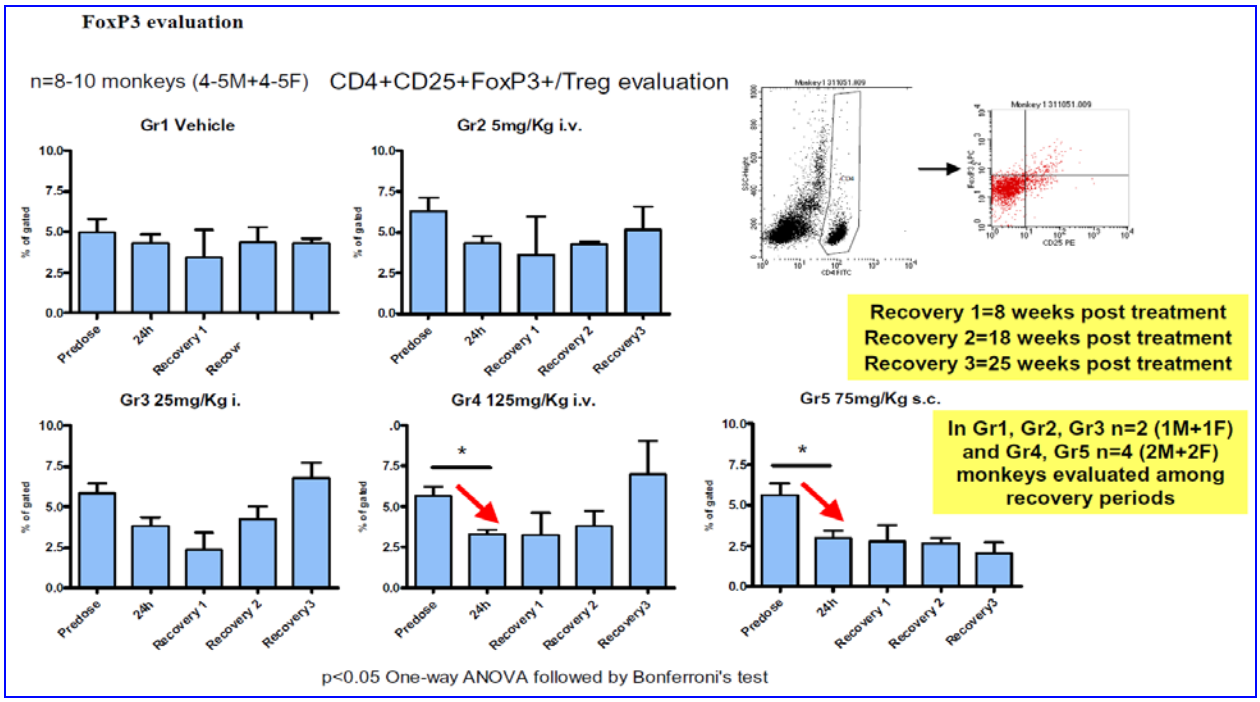


Fig. 54: *FoxP3 evaluation over time within each group - Males & Females (monkeys)*

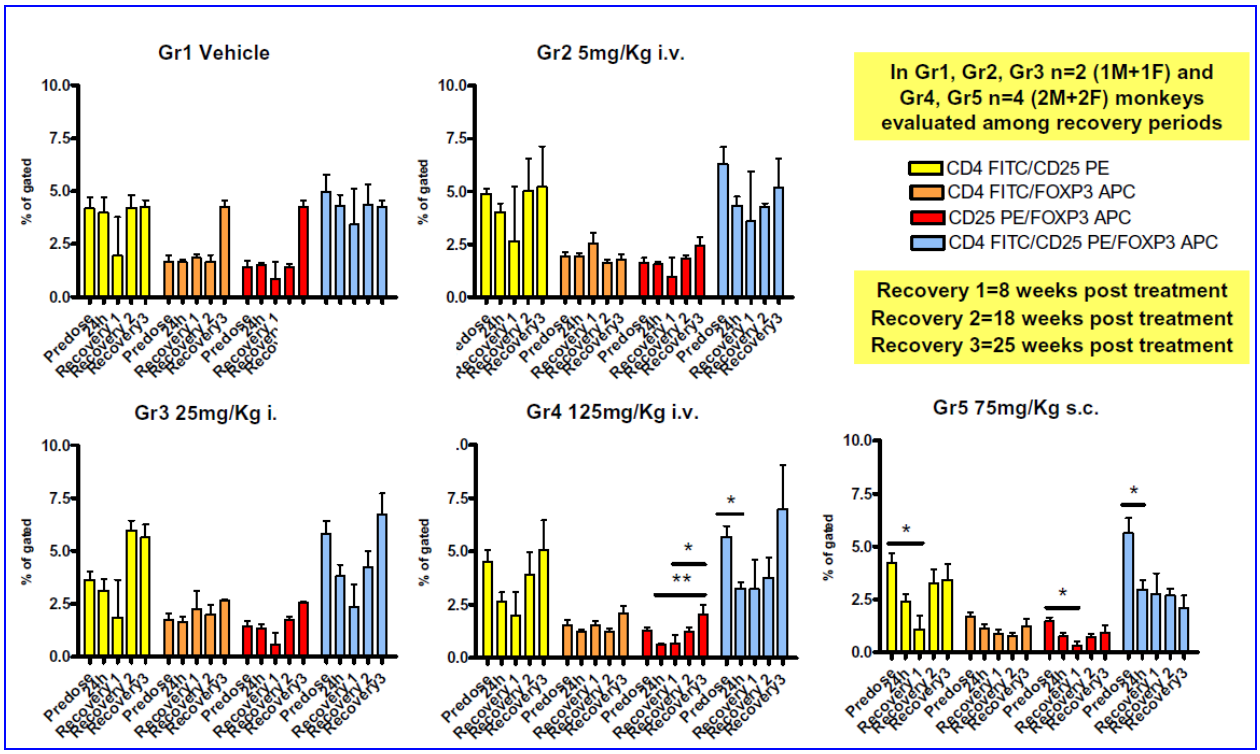


Fig.55*: FoxP3 evaluation over time within each group - Males & Females (monkeys)*

6. DISCUSSION

In this study the effects of repeated weekly administration of the test item Anti-CD25 on Cynomolgus monkeys were evaluated.

The test item was given by subcutaneous or intravenous route to 22 males and 22 females (4 animals/sex for groups 1, 2 and 3 and 5 animals/sex for groups 4 and 5) every week for 5 consecutive weeks as shown below:

Group	***Doses*** ***(mg /kg/ week)*** ***[administr. route]***	***Volume of administr.*** ***(ml/Kg)***	***Concentr. in vehicle*** ***(mg/ml)***	***No. of males***	***No. of females***	***Group color identification***
1	0 (vehicle) [IV]	2	0	4	4	white
2	5 [IV]	2	2.5	4	4	yellow
3	25 [IV]	2	12.5	4	4	green
4	125 [IV]	2	62.5	5	5	red
5	75 [SC]	2	37.5	5	5	blue

During the study general clinical observations, ophthalmological examination, ECG recordings, body temperature, body weight and food consumption measurements were carried out.

Laboratory investigations (hematology, blood chemistry and urinalysis), PD markers, NK cell activity, CRS markers, antibody determination and toxicokinetic evaluations were also performed.

One week after the last treatment 3 monkeys/sex/group were sacrificed for pathology investigations, while the remaining animals were subjected to a 28-week recovery period and then sacrificed.

At 5 mg/kg/week by intravenous route:

No clinical signs were found in any animal. ECG and ophthalmoscopy did not show changes versus basal and/or versus control group.

The histological investigations did not reveal any treatment-related variations in any animal as well as clinical pathology evaluations.

At 25 mg/kg/week by intravenous route:

No clinical signs were found in any animal. ECG and ophthalmoscopy did not show changes versus basal and/or versus control group.

The histological investigations did not reveal any treatment-related variations in any animal as well as clinical pathology evaluations.

At 125 mg/kg/week by intravenous route:

No clinical signs were found in any animal. ECG and ophthalmoscopy did not show changes vs basal and/or vs control group.

The histological investigations did not reveal any treatment-related variations in any animal as well as clinical pathology evaluations.

At 75 mg/kg/week by subcutaneous route:

No clinical signs were found in any animal. ECG and ophtalmoscopy did not show changes vs basal and/or vs control group.

The histological investigations did not reveal any systemic treatment-related variations in any animal as well as clinical pathology evaluations. The local, subcutaneous and mild reactive changes were considered due to the subcutaneous treatment procedure (needle trauma).

The antibodies determination did show that only male animals of all the treated groups (from group 2 to group 5) were found positives for the presence of binding antibodies to Anti- CD25. The antibodies presence was detected starting from week 6 of the recovery period with a mean titer of >100. The positiveness stay until the last week of the recovery.

Toxicokinetics evaluation:

Serum level increased between the first and the last treatment at all doses by both administration routes. Evaluations were carried out on data from all animals on Day 1 (first treatment) and on Day 29 (last, 5th treatment) until 168 hours after both administration routes, then extended to recovery animals.

After intravenous doses, concentrations peaked at 0.083-0.5 h, increasing between the sampling periods. Acceptable dose proportionality was found. Compound residence in the body, evaluated as MRT, was long, about 3 days on both Day 1 and Day 29, with extent to 10-15 days in animals of the recovery period. Therefore and as expected, also elimination occurred at very slow rate, recording in general half-life of about 4-5 days on Day 1 and Day 29 or longer, 10-15 days, in the profiles of the animals that completed the kinetics during the recovery period. Low clearance and small distribution volume at the steady-state were found.

In general their values were seen to decrease from Day 1 to Day 29 and to recovery weeks, suggesting compound distribution in the extracellular fluids. Accumulation after intravenous route was not observed at any doses.

Subcutaneous treatments attained their maxima levels usually within 24 h and showed concentrations increased between the first (Day 1) and the last (Day 29) administration. Exposure increased with time. The long mean residence time, about 3 days on Day 1 and Day 29, was still longer, about 15 days, in recovery animals. Similar values were recorded after IV doses. Half-life values were 6-7 days on Day1-Day29 and about 10 days in recovery animals. No significant compound accumulation was found after multiple SC administration. Bioavailability

was 108% after the first treatment and after the last treatment was 112%.

In conclusion, the *Anti-CD25 mAb* when administered by intravenous or subcutaneous route, did not result in any adverse toxicological effect neither regarding clinical observations nor clinical and morphological pathology.

However, treatment-related effects, not considered as toxicologically relevant, were observed as transient increases in serum IL-6 and reduction in serum immunoglobulins and in the percentage of blood T_{reg}-cells were observed.

7. CONCLUSION

The overall non clinical development program conducted so far in a pharmacologically responsive animal species, the cynomolgus monkey, did not reveal any overt toxicity deriving either from on-target or off-target effects of the Anti-CD25 mAb drug candidate.

There were some modulations of the pharmacodynamics observed and consistent with the molecule mode of action such as the binding and saturation of the membrane-bound ligand expressed on a minor portion of circulating CD4+cells. The down-modulation of the CD25 expression was marginal as was the production of host-anti drug antibodies, thus evaluation of the safety of the compound was not hindered. There was no evidence of any rebound effects in the recovery period.

A safety signal was however detected in the study since an increase in the plasma levels of some cytokines involved in the acute phase reaction, i.e. IL-6, was consistently observed.
This finding strongly suggests the need to evaluate further the cytokine release potential of antiCD25mAb in validated *in vitro* models with PBMC and whole blood from human subjects, either from healthy subjects and patients, across the putative range of therapeutically significant concentrations before investing in any clinical development program.

Results from these in vitro studies together with the data coming from receptor occupancy with patient blood cells and kinetic simulation will help in assessing a provisional benefit-risk for the drug candidate and defining the most likely starting dose in "first-in -human clinical trials "

8. ACKNOWLEDGMENTS

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Foremost, I would like to express my sincere gratitude to my advisors Wolfgang Kaufmann, Laura Comotto and my tutor Manuela Onidi for the support of my P.h.D study and research, for their critical review of this thesis and for their precious scientific advice, for the knowledge and for leading me working on diverse exciting projects.

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9. LIST OF ABBREVIATION

- IL-2: interleukin-2
- IL-2R: interleukin-2 receptor
- MS: multiple sclerosis
- T_{reg}: regulatory T cell
- NK: natural killer
- PTKs: protein tyrosine kinases
- TCRs: T cell receptors
- AICD: activation-induced cell death
- TNF: tumor-necrosis factor
- DNA: deoxyribonucleic acid
- ICH: international conference on harmonization
- GLP: good laboratory practice
- NOAEL: no observed adverse effect level
- IV: intravenous
- SC: subcutaneous
- PD: pharmacodynamic
- PK: pharmacokinetic
- TCR: tissue cross-reactivity
- ADA: anti-drug antibodies
- NHPs: nonhuman primates
- EFD: embryo-fetal development
- PPND: pre/post-natal development
- PHA: phytohemagglutinin
- MFI: mean fluorescence intensity
- AT: antitrypsin
- AGP: acid glycoprotein
- CRP: c-reactive protein
- PBMC: peripheral blood mononuclear cell
- IgG: immunoglobulin G
- IgM: immunoglobulin M
- i.e.: idem est

10. REFERENCES

[1] T.R. Malek, A.L. Bayer, Tolerance, not immunity, crucially depends on IL-2, Nature Rev. Immunol. 2004 (4):665-674.

[2] Y.W. He, T.R. Malek, The structure and function of c-dependent cytokines and receptors: regulation of T lymphocyte development and homeostasis. Crit. Rev. Immunol.1998 (18): 503–524.

[3] D.M. Willerford, et al. Interleukin-2 receptor chain regulates the size and content of the peripheral lymphoid compartment. Immunity 1995 (3):521–530.

[4] J. Parrish-Novak, D.C. Foster, R.D. Holly, C.H. Clegg, Interleukin-21 and the IL-21 receptor: novel effectors of NK and T cell responses. J. Leukoc. Biol. 2002 (72): 856–863.

[5] T.A. Waldmann, S. Dubois, Y. Tagaya, Contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for immunotherapy. Immunity 2001 (14):105–110.

[6] H. Suzuki, G.S. Duncan, H. Takimoto, T.W. Mak, Abnormal development of intestinal intraepithelial lymphocytes and peripheral natural killer cells in mice lacking the IL-2 receptor βchain. J. Exp. Med. 1997 (185): 499–505.

[7] M.K. Kennedy, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J. Exp. Med. 2000 (191): 771–780.

[8] T. Miyazaki, T. Taniguchi, Coupling of the IL2 receptor complex with non-receptor protein tyrosine kinases. Cancer Surv. 1996 (27): 25–40.

[9] J. Gomez, A. Gonzalez, A.C. Martinez, A. Rebollo, IL-2-induced cellular events. Crit. Rev. Immunol. 1998 (18): 185–220.

[10] L.A. Rubin, F. Galli, W.C. Greene, D.L. Nelson, G. Jay, The molecular basis for the generation of the human soluble interleukin 2 receptor. Cytokine 1990 (2): 330–336.

[11] R.J. Robb, R.M. Kutny, Structure-function relationships for the IL 2-receptor system. IV. Analysis of the sequence and ligand-binding properties of soluble Tac protein. J. Immunol. 1987 (139): 855–862.

[12] L.A. Rubin, G. Jay, D.L. Nelson, The released interleukin 2 receptor binds interleukin 2 efficiently. J. Immunol. 1986 (137): 3841–3844.

[13] Jacques Y., Le Mauff B., Boeffard F., Godard A., Soulillou J.P., A soluble interleukin 2 receptor produced by a normal alloreactive human T cell clone binds interleukin 2 with low affinity, J. Immunol., 139, pp. 2308–2316, 1987

[14] L.K. Jung, T. Hara, S.M. Fu, Detection and functional studies of p60-65 (Tac antigen) on activated human B cells. J. Exp. Med. 1984 (160): 1597–1602.

[15] T.A. Fehniger, M.A. Cooper, G.J. Nuovo, M. Cella, F. Facchetti, M. Colonna, M.A. Caligiuri, $CD56^{bright}$ natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. Blood, 2003 (101):3052–3057

[16] R.A. Seder, W.E. Paul, Acquisition of lymphokine-producing phenotype by CD4+ T cells, Annu. Rev. Immunol. 1994 (12): 635–673.

[17] X. Paliard, R. de Waal Malefijt, H. Yssel, D. Blanchard, I. Chretien, J. Abrams, J. de Vries, H. Spits, Simultaneous production of IL-2, IL-4, and IFN-gamma by activated human CD4+ and CD8+ T cell clones. J. Immunol. 1988 (141): 849–855.

[18] S. Sakaguchi, Rev. Immunol. 2004 (22): 531–562.

[19] Q. Tang, et al. J. Immunol. 2003 (171):3348-3352.

[20] T.R. Malek, L.A. Bayer. Nature Rev. Immunol. 2004(4): 665-674.

[21] K.A. Smith, T-cell growth factor. Immunol. Rev. 1980 (51): 337–357.

[22] Y.W. He, H. Nakajima, W. J. Leonard, B. Adkins, T. R. Malek, The common γ-chain of cytokine receptors regulates intrathymic T cell development at multiple stages. J. Immunol. 1997 (158): 2592–2599.

[23] T.R. Malek, G. Ortega, J.P. Jakway, C. Chan, E.M. Shevach, The murine IL 2 receptor. II. Monoclonal anti-IL 2 receptor antibodies as specific inhibitors of T cell function *in vitro*. J. Immunol. 1984 (133)**:** 1976–1982.

[24] G.A. Koretzky, R.P. Daniele, W.C. Greene, P.C. Nowell, Evidence for an interleukin-independent pathway for human lymphocyte activation. Proc. Natl Acad. Sci. USA 1983 (80): 3444–3447.

[25] T.R. Malek, A. Yu , P. Scibelli , M.G. Lichtenheld, E.K. Codias, Broad programming by IL-2 receptor signaling for extended growth to multiple cytokines and functional maturation of antigen-activated T cells. J. Immunol. 2001 (166):1675–1683.

[26] J. Cote-Sierra, et al. Interleukin 2 plays a central role in T_H2 differentiation. Proc. Natl Acad. Sci. USA 2004 (101)**:** 3880–3885.

[27] L. Van Parijs, A.K. Abbas, Homeostasis and self-tolerance in the immune system: turning lymphocytes off. Science 1998 (280): 243–248.

[28] L. Zheng, C.L. Trageser, D.M. Willerford, M.J. Lenardo, T cell growth cytokines cause the superinduction of molecules mediating antigen-induced T lymphocyte death. J. Immunol. 1998 (160): 763–769.

[29] H. Sepulveda, A. Cerwenka, T. Morgan, R.W. Dutton, CD28, IL-2-independent costimulatory pathways for CD8 T lymphocyte activation. J. Immunol. 1999 (163): 1133–1142.

[30] P.S. Changelian, et al. Prevention of organ allograft rejection by a specific Janus kinase 3 inhibitor. Science 2003 (302): 875–878.

[31] O. Lantz, I. Grandjean , P. Matzinger, J. Di Santo. γ chain required for naive $CD4^+$ T cell survival but not antigen proliferation. Nature Immunol. 2000 (1): 54–58.

[32] M.K. Levings, R. Sangregorio, M.G. Roncarolo. Human $CD25^+CD4^+$ T regulatory cells suppress naive and memory T cell proliferation and can be expanded *in vitro* without loss of function. J. Exp. Med. 2001 (193): 1295–1302.

[33] C. Baecher-Allan, J.A. Brown, G.J. Freeman , D.A. Hafler. $CD4^+CD25^{hi}$ regulatory cells in human peripheral blood. J. Immunol. 2001 (167): 1245–1253.

[34] C.M. Roifman. Human IL-2 receptor chain deficiency. Pediatr. Res. 2000 (48).

[35] M.B. Atkins. Interleukin-2: clinical applications. Semin. Oncol. 2002 (29).

[36] J.C. Yang, et al. Randomized study of high-dose and low-dose interleukin-2 in patients with metastatic renal cancer. J. Clin. Oncol. 2003 (21): 3127–3132.

[37] M. Murakami, A. Sakamoto, J. Bender, J. Kappler, P. Marrack. CD25+CD4+ T cells contribute to the control of memory CD8+ T cells. Proc. Natl Acad. Sci. USA 2002 (99): 8832–8837.

[38] R.T. Davey, et al. Immunologic and virologic effects of subcutaneous interleukin 2 in combination with antiretroviral therapy: a randomized controlled trial. JAMA 2000 (284):183–189.

[39] I. Sereti, et al. IL-2 induced CD4+ T-cell expansion in HIV-infected patients is associated with long-term decreases in T-cell proliferation. Blood 2004 (104): 775–780.

[40] R.S. McHugh, E.M. Shevach, Depletion of CD4+CD25+ regulatory T cells is necessary, but not sufficient, for induction of organ-specific autoimmune disease. J. Immunol. 2002 (168): 5979–5983.

[41] F.M. Foss, Interleukin-2 fusion toxin: targeted therapy for cutaneous T cell lymphoma. Ann. NY Acad. Sci. 2001 (941): 166–176.

[42] R.P. Sutmuller, et al. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25+ regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. J. Exp. Med. 2001 (194): 823–832.

[43] F. Ichihara, et al. Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. Clin. Cancer Res. 2003 (9): 4404–4408.

[44] T.R. Malek, A. Yu, V. Vincek, P. Scibelli, L. Kong. CD4 regulatory T cells prevent lethal autoimmunity in IL-2R-deficient mice. Implications for the no redundant function of IL-2. Immunity 2002 (17): 167–178.

[45] A.C. Church, et al. Clinical advances in therapies targeting the interleukin-2 receptor. QJM 2003 (96):91–102.

[46] A.M. Shapiro, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N. Engl. J. Med. 2000 (343): 230–238.

[47] R. Gold, G.Giovannoni, K. Selmaj, E. Havrdova, X. Montalban, E-W. Radue, D. Stefoski, R. Robinson, K. Riester, J.Rana, J. Elkins, G. O'Neill. Daclizumab high-yield process in relapsing-remitting multiple sclerosis: a randomised, double-blind, placebo-controlled trial, Lancet 2013 (381): 2167-75.

[48] M.P. Sormani, L. Bonzano, L. Roccatagliata, et al. Surrogate endpoints for EDSS worsening in multiple sclerosis. A meta-analytic approach. Neurology 2010 (75): 302–09.

[49] B. Bielekova, N. Richert, T.Howard, G. Blevins, S. Markovic-Plese, J. McCartin, J.A. Frank, J. Wurfel, J. Ohayon, T.A. Waldmann, H.F. McFarland, R. Martin; Humanized anti-CD25 (daclizumab) inhibits disease activity in multiple sclerosis patients failing to respond to interferon β. Pnas 2004 (110): 8705-8708.

[50] J.P. Sheridan, Y. Zhang, K. Riester, et al. Intermediate-affinity interleukin-2 receptor expression predicts $CD56^{bright}$ natural killer cell expansion after daclizumab treatment in the choice study of patients with multiple sclerosis. Mult Scler. 2011 (17): 1441–48.

[51] B. Bielekova, M. Catalfamo, S. Reichert-Scrivner, et al. Regulatory $CD56^{bright}$ natural killer cells mediate immunomodulatory effects of IL-2Ralpha-targeted therapy (daclizumab) in multiple sclerosis. Proc. Natl. Acad. Sci. USA 2006 (103): 5941–46.

[52] ICH S6 Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals; 1997

[53] ICH S5(R2) Detection of Toxicity to Reproduction for Medicinal Products and Toxicity to Male Fertility; 1993

[54] ICH S1A The Need for Carcinogenicity Studies of Pharmaceuticals; 1995

[55] ICH M3(R2) Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals; 2009

[56] ICH S9 Nonclinical Evaluation for Anticancer Pharmaceuticals; 2008

[57] N. Pentsuk, J.W. Van der Laan. An interspecies comparison of placental antibody transfer: new insights into developmental toxicity testing of monoclonal antibodies. Dev. Reprod. Toxicol. 2009 (86): 328-344.

[58] P. Jarvis, S. Srivastav, E. Vogelwedde, J. Stewart, T. Mitchard, G. Weinbauer. The cynomolgous monkey as a model for developmental toxicity studies: variability of pregnancy losses, statistical power estimates, and group size considerations. Dev. Reprod. Toxicol. 2010 (89): 175-187.

[59] J.M. Ellery, P.J. Nicholls. Alternative signalling pathways from the interleukin-2 receptor. Cytokine Growth Factor. Rev. 2002 13(1): 27-40.

[60] T.A. Waldmann, et al. The IL-2/IL-2 receptor system: a target for rational immune intervention. Immunol. 1993 14(6): 264-70.

[61] J.C. Morris, T.A. Waldmann. Advances in interleukin 2 receptor targeted treatment. Ann. Rheum. Dis. 2000 59(1): 109-14.

[62] T.A. Waldamann, J.D. White, C.K. Goldman, L. Top, A. Grant, R. Bamford, E. Roessler, I.D. Horak, S. Zaknoen, C. Kasten-Sportes, et al. The interleukin-2 receptor: a target for monoclonal antibody treatment of human T-cell lymphotrophic virus I-induced adult T-cell leukaemia. Blood. 1993 82(6): 1701-12.

[63] J.L. De Jong, N.L. Farner, M.B. Widmer, J.G. Giri, P.M. Sondel. Interaction of IL-15 with the shared IL-2 receptgor beta and gamma c subunits. The IL-15/beta/gamma c receptor-ligand complex is less stable than the IL-2/beta/gamma c receptor-ligand complex. J. Immunol. 1996 156(4): 1339-48.

[64] B. Kuttler, K. Rosing, M. Lehmann, J. Brock, H.J. Hahn, Prevention of autoimmune but not allogeneic destruction of grafted islets by different therapeutic stragegies. J. Mol. Med. 1999 77(1): 229-9.

[65] T.R. Jones, J. Ha, M.A. Williams, A.B. Adams, M.M. Durham, P.A. Rees, S.R. Cowan, T.C. Pearson, C.P. Larsen. The Role of the IL-2 pathway in costimulation blockade-resistant rejection of allografts. J. Immunol. 2002. 168(3): 1123-30.

[66] C. Anasetti, J.A. Hansen, T.A. Waldmann, F.R. Appelbaum, J. Davis, H.J. Deeg, K. Doney, P.J. Martin, R. Nash, R. Storb, and et al. Treatment of acute graft-versus-host disease with humanized anti-Tac: an antibody that binds to the interleukin-2 receptor. Blood. 1994 84(4): 1320-27.

[67] J.D. Silvertown et al. Cloning sequencing and characterization of lentiviral- mediated expression of rhesus macaque (Macaca Mulatta) interleukin- 2 receptor alpha cDNA. Dev. Com. Immunol. 2005 29(11):989-1002.

[68] B. Bielekova, R. Martin, Multiple Sclerosis: Immunotherapy. Curr. Treat. Options Neurol. 1999 (1): 201–219.

[69] T. Waldmann, et al. Interleukin-2 , interleukin- 15, and their receptors. Int. Rev. Immunol. 1998 (16): 205–226.

[70] B. Bielekova et al. Humanized anti- CD25 (daclizumab) inhibits diseases activity in Multiple Sclerosis patients failing to respond to interferon beta. Proc. Natl. Acad. Sci. 2004 101(23):8705-08.

[71] C. Queen, et al. A humanized antibody that binds to the interleukin-2 receptors. Proc. Natl. Acad. Sci. USA 1989 (86): 10029–33.

[72] J. Huan, et al. Decreased FoxP3 levels in Multiple Sclerosis patients. J. of Neurosc. Res. 2005 (81): 45-52.