Interleukin-10 derived from macrophages and/or neutrophils regulates the inflammatory response to LPS but not the response to CpG oligonucleotides

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Abstract

Interleukin-10 (IL-10) is an important regulator of immune responses secreted by different cell types. We have previously shown that mice with selective inactivation of the *IL-10* gene in T cells suffer from deregulated T cell responses similar as observed in *IL-10*$^{-/-}$ animals. Unlike *IL-10*$^{-/-}$ mice, however, T cell-specific mutants do not mount an enhanced innate response to LPS, which must, therefore, be subject to control by IL-10 from non-T cells. Herein we show that subcutaneous injection of LPS, causing a moderate local inflammation in WT and T cell-specific *IL-10* mutant mice, resulted in augmented inflammatory infiltration and extensive tissue necrosis in mice with deficiency for IL-10 in macrophages and neutrophils. Correspondingly, we observed an enhanced sensitivity of the macrophage/neutrophil-specific *IL-10* mutants to systemic LPS exposure when compared with WT animals. In contrast, the inflammatory response of these mutants to CpG oligodeoxynucleotides was not different from that of WT mice. While *IL-10*$^{-/-}$ mice developed massive inflammation, necrosis and increased serum cytokine levels after subcutaneous CpG injection, only moderate responses were observed in macrophage/neutrophil-specific *IL-10* mutant and WT mice. These results show that different innate responses can be subject to control by IL-10 from different cellular sources.

1. Introduction

The innate immune system senses microbial infection by receptors, which detect conserved pathogen-associated molecular patterns and mediate inflammatory responses. These responses serve as a first line of defense against invading pathogens but are also of pivotal importance for the induction and regulation of adaptive immunity. An important family of pattern recognition receptors are the Toll like receptors (TLRs), which recognize a diverse spectrum of ligands including nucleic acids, lipids and proteins (reviewed in [1], [2]). TLR4 recognizes lipopolysaccharide (LPS) of Gram-negative bacteria and mediates secretion of proinflammatory cytokines, most notably, TNF-α, IL-12 and IFN-γ(reviewed in [3]). Furthermore, TLR4 stimulation induces dendritic cell maturation and B cell proliferation [3]. TLR9 triggers an inflammatory response upon recognition of unmethylated CpG motifs in bacterial or viral DNA, which are less common in mammalian DNA (reviewed in [4]). TLR9 ligation is a stimulus for maturation of dendritic cells as well as B cell proliferation and Ig secretion. Both cell types, as well as murine macrophages, up-regulate molecules involved in antigen presentation and secrete a Th1 like pattern of cytokines as a result of TLR9 stimulation. In contrast, plasmacytoid dendritic cells respond to TLR9 ligation by secretion of large amounts of IFN-α [4].

While TLR responses are of vital importance for the control of multiple pathogens, uncontrolled TLR stimulation can induce severe damage to host tissue. The most prominent example of TLR-mediated immunopathology is endotoxic shock [3], which results from an overwhelming production of pro-inflammatory mediators, in particular TNF-α, resulting in systemic microvascular damage. IL-10 is an immunoregulatory cytokine, which limits immune responses and minimizes immunopathology (reviewed in [5]). In *IL-10*$^{-/-}$ mice [6], deregulated Th1 responses

result in the spontaneous development of inflammatory bowel disease [6] as well as severe immunopathology upon infection with a number of different pathogens [7]-[10]. The importance of IL-10 for the control of innate responses was demonstrated by an increased sensitivity of IL-10-deficient animals to lipopolysaccaride (LPS). *IL-10*$^{-/-}$ mice succumb to septic shock following the intraperitoneal administration of 20-fold lower doses of LPS as compared to WT animals [11]. Likewise, the response of the skin to tetradecanoylphorbol-acetate (TPA) containing irritants, which is a function of cutaneous innate immunity, is clearly enhanced in IL-10-deficient mice in comparison to WT mice [12]. Thus, innate responses as well as Th1 responses are subject to regulation by IL-10.

We have previously shown that mice with a T cell-specific inactivation of the *IL-10* gene reproduce the deregulated T cell responses of completely IL-10-deficient animals but develop normal innate responses to LPS or skin irritation [13]. These innate responses must, therefore, be subject to control by IL-10 derived from non-T cells. Here we report on the analysis of innate responses in mice with a deficiency for IL-10 in macrophages and neutrophils.

2. Results

IL-10^{FL} mice on a mixed 129/C57BL/6 background have been described earlier [13]. Since alterations of immune responses in IL-10-deficient mice depend on the genetic background (for example see [14]), we generated an additional *IL-10*^{FL} line by re-targeting the *IL-10* gene in C57BL/6-derived Bruce4 embryonic stem cells using the same targeting vector. This new *IL-10*^{FL} line was used for the experiments described below.

Selective inactivation of the IL-10 gene in macrophages and neutrophils

IL-10^{FL} animals were crossed to C57BL/6 *lysM-Cre* transgenic mice, which express the Cre recombinase in macrophages and neutrophilic granulocytes [15]. Efficiency and specificity of Cre-mediated deletion were verified by Southern blot analysis of DNA extracted from FACS-sorted cell populations. Complete deletion of the loxP-flanked fragment was found in F4/80^{+} macrophages from the peritoneal cavity and in Gr1^{+} neutrophilic granulocytes from the spleen of *IL-10*$^{FL/FL}$ *lysM-Cre*$^{+}$ mice (Figure 1). In contrast, no or only insignificant deletion was observed in CD19^{+} splenic B cells, CD3^{+} T cells, CD11c+ splenic dendritic cells or in tail biopsies from the same animals. Thus, inactivation of the *IL-10* gene in *IL-10*$^{FL/FL}$ *lysM-Cre*$^{+}$ mice is efficient in macrophages as well as in neutrophilic granulocytes and was not detected in other cell types by Southern blot analysis.

Comparison of the LPS response in different IL-10 mutant mice

In order to test the importance of IL-10 derived from different cell types for the control of the local inflammatory response to LPS, we first established a regimen for subcutaneous LPS administration in *IL-10*$^{-/-}$ and WT mice. 100 µl of 0.4 mg LPS/ml in NaCl were injected s.c. once daily at the same site (flank) until a difference in the local response between the two groups became macroscopically evident. After three injections, all *IL-10*$^{-/-}$ animals (n=4) but none of the WT mice (n=4) developed a solid subcutaneous swelling. Histologically, the lesion was characterized by a massive infiltration of macrophages and neutrophils, edema and an extensive necrosis of epidermis, dermis and the panniculus carnosus (i.e. the muscle layer of murine skin) in all IL-10-deficient mice as demonstrated by H&E stained paraffin sections (Figure 2). In contrast, only relatively moderate inflammatory changes without any signs of tissue necrosis were observed at the injection site in all WT mice. We conclude that subcutaneous injections of LPS and subsequent analysis of the injected tissue by histology is a convenient means to assess the LPS sensitivity of mutant mice.

Upon s.c. administration of LPS (daily injections on three successive days, see above), the *IL-10*$^{FL/FL}$ *lysM-Cre*$^{+}$ mice (n=5) developed an enhanced inflammatory infiltration with extensive necrosis similar to the local response in mice with complete IL-10 deficiency (Figure 2) while T cell-specific *IL-10* mutant mice (*IL-10*$^{FL/FL}$ *CD4-Cre*$^{+}$ [13], n=5) showed the same moderate local response as control animals (Figure 2),

We have published earlier that, in contrast to *IL-10*$^{-/-}$ mice, which succumb to low doses of LPS administered i.p. due to overproduction of pro-inflammatory cytokines [11], the LPS sensitivity of mice with selective IL-10 deficiency in T cells is the same as in WT mice [13]. In order to further characterize the LPS sensitivity of mice

with a macrophage/neutrophil-specific deficiency for IL-10 production, LPS was injected i.p. in *IL-10*$^{-/-}$, *IL-10*$^{FL/FL}$ *lysM-Cre*$^{+}$, *IL-10*$^{FL/FL}$ *CD4-Cre*$^{+}$ mice as well as *Cre*$^{-}$ littermate and wt controls. Survival was monitored for 48 hours (Figure 3 A). 23 out of 25 control mice and nine out of 10 *IL-10*$^{FL/FL}$ *CD4-Cre*$^{+}$ mice survived this treatment, whereas all *IL-10*$^{-/-}$ animals succumbed by 24 hours post injection. Among 10 *IL-10*$^{FL/FL}$ *lysM-Cre*$^{+}$ mice, six had died by day 2 after injection. Serum samples were obtained six hours after the i.p. injection and were assayed for the concentrations of proinflammatory cytokines (Figure 3 B). While *IL-10*$^{-/-}$ mice showed a substantial increase in the serum levels of TNF-α, IL-12 and IFN-γ, low or undetectable concentrations of these cytokines were determined in the sera of the controls and the T cell-specific IL-10 mutant mice. The cytokine levels of the *IL-10*$^{FL/FL}$ *lysM-Cre*$^{+}$ mice were significantly higher than those of the controls but did not reach the levels of IL-10$^{-/-}$ mice.

These data show that mutants with a selective deficiency for IL-10 secretion in macrophages and neutrophils are characterized by enhanced sensitivity to LPS when compared with wt controls. However, the sensitivity of the macrophage/neutrophil-specific mutant mice to systemic LPS exposure is clearly less pronounced than the LPS sensitivity of mice with complete IL-10 deficiency.

Comparison of the response to CpG-DNA in different IL-10 mutant mice

In order to characterize the local inflammatory response to TLR9 stimulation, a CpG-oligodeoxynucleotide ([4], 2.5 µg in 50 µl NaCl) was injected s.c. once daily at the same site (flank). After 5 days, all *IL-10*$^{-/-}$ mice (n=9) but none of the WT animals had developed a solid subcutaneous swelling at the injection site. Mice were sacrificed at day 6 and the injection site was excised and analysed by histology. In WT or *Cre*$^{-}$ control animals (n=7), the CpG injections resulted in a moderate belt

shaped inflammatory infiltrate along and below the panniculus carnosus as demonstrated by H&E stained paraffin sections (Figure 4). Neither tissue necrosis nor inflammatory edema was detectable. The infiltrate was composed primarily of macrophages as demonstrated by immunostaining for F4/80 (not shown). No infiltrating neutrophilic granulocytes were detectable by staining for the neutrophil-specific enzyme chloroacetate esterase. In marked contrast, the lesions of all *IL-10*$^{-/-}$ mice showed conspicuous edema, massive infiltration by macrophages and also numerous neutrophilic granulocytes and extensive necrosis of epidermis, dermis and panniculus carnosus (Figure 4). The necrotic area extended over three to five medium power (x100) fields (not shown). These data show an increased sensitivity of IL-10-deficient mice to CpG oligonucleotides as compared to WT animals, which had not been described before. Furthermore this result demonstrates that, like the local injection of LPS, also subcutaneous administration of TLR9 ligands followed by histological evaluation of the tissue surrounding the injection site is a useful system to evaluate the innate responsiveness of mice.

The local response of *IL-10*$^{FL/FL}$ *CD4-Cre*$^{+}$ mice (n=4) to s.c. CpG administration (daily injections on five successive days, see above) was as in WT mice with a moderate infiltration of macrophages but no infiltrating granulocytes and without necrosis or inflammatory edema (Figure 4). To our surprise, however, although murine macrophages in contrast to human macrophages express TLR9 and are responsive to CpG oligonucleotides [16, 17], the *IL-10*$^{FL/FL}$ *lysM-Cre*$^{+}$ (n=7) mice also showed only a moderate inflammatory local response to CpG oligonucleotides without granulocyte infiltration or necrosis (Figure 4). The response of these animals could not be distinguished from that of WT animals.

Serum was sampled from *IL-10*$^{-/-}$, *IL-10*$^{FL/FL}$ *lysM-Cre*$^{+}$, *IL-10*$^{FL/FL}$ *CD4-Cre*$^{+}$ and control mice after 5 days of successive s.c. injections of CpG oligonucleotides and

assayed for concentrations of pro-inflammatory mediators. Corresponding to the results of the histological analysis of the lesion at the site of injection, we measured increased levels of TNF-α, IL-6 and IFN-γ in *IL-10*$^{-/-}$ animals when compared to control mice (Figure 5). Cytokine levels determined for the mice with macrophage/neutrophil-specific and the mice with T cell-specific IL-10 deficiency were in the same low range as those determined for WT mice (Figure 5). Likewise, *IL-10*$^{-/-}$ mice receiving mock CpG oligonucleotides did not show increased concentrations of the pro-inflammatory mediators.

Collectively, these data show that IL-10 is important for the control of the TLR9 response to CpG. Selective deficiency for IL-10 in macrophages and neutrophilic granulocytes, however, does not result in an enhanced sensitivity to subcutaneously administered CpG-oligonucleotides.

Murine macrophages secrete IL-10 upon stimulation with CpG-DNA.

The finding that selective inactivation of the *IL-10* gene in macrophages and neutrophils in mice does not result in an enhanced inflammatory response to subcutaneous injections of CpG DNA raises the question whether murine macrophages can, in principle, secrete IL-10 upon exposure to CpG oligonucleotides. We therefore sorted F4/80-positive macrophages and CD19-positive B cells from spleen cell suspensions of wt mice by FACS and cultivated these cells in the presence of CpG oligonucleotides. After 24 and 48 hours, supernatants were assayed for IL-10 concentration (Figure 6). While no IL-10 production was detected in unstimulated or mock-CpG stimulated cultures, B cells as well as macrophages produced significant amounts of IL-10 in response to stimulation with CpG DNA. These data show, that murine splenic macrophages are capable of producing IL-10 in reponse to TLR9 ligation.

Inactivated IL-10 alleles can be detected in skin lesions of *IL-10$^{FL/FL}$ lysM-Cre^{+}* mice.

In order to rule out that the macrophages accumulating at the site of CpG injection represent a particular subset which does not activate the lysM-promotor and, therefore, does not incativate the *IL-10* gene in *IL-10$^{FL/FL}$ lysM-Cre^{+}*, we compared the ratio of loxP-flanked and deleted *IL-10* alleles in untreated and CpG-injected skin of *IL-10$^{FL/FL}$ lysM-Cre^{+}* mice by Southern blot analysis (Figure 7). The signal for the deleted allele was clearly more intense for the samples of CpG-treated skin. Since the most prominent change in the cellular composition of the cutaneous tissue upon CpG administration is the influx of a small number (relative to the overall cellularity of the tissue) of macrophages (see above), we conclude that at least a major fraction of these cells must carry inactivated IL-10 alleles.

3. Discussion

IL-10 is an important regulator of TLR-mediated innate responses. IL-10-deficient mice are highly sensitive to systemically administrated LPS [11] and IL-10 also inhibits the TLR9 response to bacterial DNA or CpG oligonucleotides [18], [19]. Here we report that the innate responses to LPS and CpG oligonucleotides are regulated by IL-10 derived from different cell types. Repetitive subcutaneous injection of both TLR ligands in WT mice resulted in a macrophage dominated inflammatory infiltrate but little tissue damage. In contrast to the WT animals, both responses were enhanced in complete IL-10 deficiency resulting in a more massive leukocyte infiltration and necrosis of the injected tissue. The difference between WT and mutant mice in the local response to these TLR ligands was distinct in all animals analyzed and allows an unambiguous assignment of the correct genotypes in a blinded setting. Therefore, the local application of LPS and CpG oligonucleotides with subsequent histological evaluation is a simple and reliable means to assess the reactivity of mouse mutants to these and probably also other TLR ligands.

The TLR4-mediated local response to LPS of mice with a selective defect for IL-10 production in macrophages and neutrophilic granulocytes resulted in enhanced leukocyte infiltration compared with control mice and in the development of tissue necrosis which did not occur in the controls. These changes were similar to those we observed in *IL-10*$^{-/-}$ mice. Consistent with the deregulated LPS response in the macrophage/neutrophil-specific *IL-10* mutant mice, we also showed a deregulation of the systemic response to an intraperitoneal administration of LPS as demonstrated by reduced survival and increased serum levels of pro-inflammatory cytokines in comparison with control mice. However, compared with *IL-10*$^{-/-}$ animals, the deregulation of the systemic LPS response of the mice with macrophage/neutrophil-specific inactivation of the *IL-10* gene was signigicantly less pronounced, which

demonstrates that also cell types other than macrophages and neutrophils must contribute to the control of the TLR4 response by secretion of IL-10. Our experiment does not allow a distinction between IL-10 production in macrophages and neutrophils since the *lysM-Cre* transgenic line expresses Cre in both of these cell types.

In contrast to the TLR-4 mediated response of the macrophage/neutrophil-specific mutant mice to LPS, the TLR9-mediated response of these mice to CpG oligonucleotides was comparable to that of WT animals. In T cell-specific *IL-10* mutant mice, the response to both stimuli was similar to that in control mice. Thus, IL-10 derived from myeloid cells, but not T cell-derived IL-10, is important for the control of the response to LPS, and cell types other than macrophages, neutrophilic granulocytes or T cells regulate the response to bacterial DNA by secretion of IL-10.

The finding that macrophage/neutrophil-derived IL-10 contributes to the control of the LPS response is not unexpected. Macrophages produce IL-10 in response to LPS [20]-[22]. By secretion of IL-10, macrophages down regulate their own activation and pro-inflammatory mediator secretion in an autokrine loop [20]. Cultivation of human monocytes in the presence of macrophage colony-stimulating factor results in the differentiation of a macrophage population, which upon stimulation with LPS secretes IL-10 but no Th1-promoting cytokines [21]. In contrast to IL-12-producing macrophages differentiated with granulocyte-macrophage colony-stimulating factor, these cells did not support the control of intracellular pathogens. Furthermore, treatment of human and murine monocytes with IL-4 or glucocorticoids results in the differentiation of an additional subset of macrophages which is characterized by a lack of pro-inflammatory capacity and prominent IL-10 production (reviewed in [23]). Thus, specialized IL-10 secreting subsets of macrophages may exert immuno-

regulatory functions *in vivo* [21]. In addition, also the production of relevant amounts of IL-10 by neutrophilic granulocytes cannot be excluded [5], [24].

However, the finding that selective inactivation of the *IL-10* gene in macrophages and neutrophils is irrelevant for the control of the local response to CpG oligonucleotides was unexpected. Murine plasmacytoid and myeloid dendritic cells [25], mast cells (reviewed in [26]) as well as B cells [27], [28] express TLR9 and respond to bacterial DNA. Sensitivity to bacterial DNA and CpG oligonucleotides has been demonstrated for a murine macrophage cell line and for macrophages differentiated from mouse bone marrow *in vitro* [16], [17]. We show in the present paper that murine splenic macrophages secrete IL-10 in response to stimulation with CpG oligonucleotides. Thus, although macrophage subsets may differ in TLR9 expression, it seems likely that the macrophages recruited into the subcutaneous tissues after CpG injection are directly responsive to the TLR stimulus and that their response to CpG DNA entails IL-10 production.

How can we explain our finding that macrophage-derived IL-10 does not significantly contribute to the control of the local inflammatory response to CpG oligonucleotides? One could speculate that in contrast to LPS, CpG DNA might recruit a subset of macrophages into the tissue which does not activate the lysM-promotor and thus does not inactivate the loxP-flanked *IL-10* gene in *IL-10*$^{FL/FL}$ *lysM-Cre*$^{+}$ mice. However, the accumulation of cells carrying inactivated *IL-10* alleles could be demonstrated in the CpG-injected tissue by Southern blot analysis.

The most likely explanation for the lack of a demonstrable effect of macrophage/neutrophil-specific IL-10 deficiency on the local CpG response is that other cell types secrete significant amounts of IL-10 in response to CpG exposure, which can fully compensate for a defective IL-10 production by macrophages.

In the future, we will address the question of the source of IL-10 important for the control of TLR9 responses by cell type-specific deletion of the *IL-10* gene in B cells, mast cells or dendritic cells.

4. Material and Methods

Reagents

LPS from *Escherichia coli* serotype 055:B5 (Sigma-Aldrich) dissolved in sterile saline was used for s.c. and i.p. injections. The phosphorothioate CpG-oligodeoxynucleotide 1826, 5'-TCC ATG ACG TTC CTG ACG TT-3', and the mock oligodeoxynucleotide 1982, 5'-TCC AGG ACT TCT CTC AGG TT-3' (Metabion, Germany), were injected s.c. in sterile saline.

Mice and genotyping

All animals used were on the C57BL/6 background. *IL-10*^{FL} animals were crossed to lysM-*Cre* [15] or *CD4-Cre* transgenic mice [29]. Southern blot analysis was performed as described earlier [6], [13]. The mice were kept in a specific pathogen free facility. All animal experimentation was done in accordance with institutional guidelines.

Cell sorting

For the determination of specificity and efficiency of Cre-mediated deletion spleen cell suspensions were subjected to erythrocyte lysis using ammonium chloride. T cells, B cells, dendritic cells, neutrophilic granulocytes and macrophages were immunostained with anti-CD3, anti-CD19, anti-CD11c (PharMingen) and anti-Gr-1 (eBioscience) antibodies, respectively, and separated with a MoFlow cell sorter (Cytomation). F4/80-positive macrophages (antibody from Caltag Laboratories) were sorted from peritoneal lavage fluid. The purity of the sorted cell populations

ranged between 92-98%. DNA was extracted from the isolated cells for Southern blot analysis as described previously [13].

For *in vitro* stimulation experiments, macrophages and B cells were sorted from spleen cell suspensions as described above. The purity of the macrophage and the B cell preparations was 95% and 92%, respectively.

In vitro stimulation of macrophages and B cells

FACS-sorted cell populations were incubated in 96 well plates at a density of $2x10^5$ cells in 0.2 ml RPMI-1640 medium supplemented with 10% FCS with either 5 µg/ml LPS, 2.5 µg/ml CpG oligodeoxynucleotide 1826 or the mock oligonucleotide 1982. After 24 and 48 hours, IL-10 concentrations in culture supernatants were measured by ELISA (R&D Systems).

Serum cytokine determination

Blood was sampled from the tail vein and undiluted serum was analysed by Cytometric Bead Array (BD Biosciences) according to the manufacturer's instructions.

Histology

For immunohistochemical staining of macrophages, paraffin sections were incubated with rat anti-mouse F4/80 (MCA497GA, Serotec, Germany) at 4°C overnight. The section was then incubated with biotin-labelled polyclonal rabbit anti-rat Ig (DakoCytomation, Germany) for 30 min followed by incubation with streptavidin-conjugated horseradish peroxidase (30 min) and aminoethyl carbazole (AEC) as chromogen (10 min). Nuclei were counterstained with hematoxylin. The

naphthol-AS-D-chloroacetate esterase reaction was performed according to standard procedures (IHC world, online information center for histochemistry)**.**

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Figure legends

Figure 1. Cell type-specific deletion of the *IL-10* gene in macrophages and neutrophilic granulocytes from *IL-10*$^{FL/FL}$ *lysM-Cre*$^{+}$ mice. Southern blot analysis of DNA extracted from FACS-sorted cell populations and tail tissue after PstI digest. F4/80^{+} macrophages were sorted from peritoneal lavage fluid. Gr1^{+} neutrophilic granulocytes, CD11c^{+} dendritic cells, CD3^{+} T cells and CD19^{+} B cells were sorted from splenocyte suspensions. FL or D designate the *loxP*-flanked or the deleted allele, respectively.

Figure 2. Local inflammatory response of *IL-10* mutant and WT mice to repetitive subcutaneous injection of LPS. 100 µl of a solution of 0.4 mg LPS/ml in NaCl were injected s.c. daily at the same site (flank) for three days. Tissue was sampled on day 4 and paraffin sections were stained with H&E. Note the belt shaped inflammatory infiltrate (marked by vertical arrows) below the panniculus carnosus (muscle layer of murine skin, ✰) in WT mice (A). Vital keratinocytes of surface and follicular epithelium display large nuclei and abundant cytoplasm. *IL-10*$^{-/-}$ animals (B) developed a more intense inflammatory infiltration and extensive necrosis of epithelial structures as well as necrosis of dermis and panniculus carnosus (✰). Note that only shadows of keratinocytes with condensed nuclei remain and that most of the epidermis is lost. The overview shows the margin of a large necrotic area extending over 3-5 medium power (x100) fields. Necrotic skin is marked by the horizontal arrow. T cell-specific IL-10 mutant mice (*IL-10*$^{FL/FL}$ *CD4-Cre*$^{+}$, C) showed a similar response as observed in WT mice without tissue necrosis while macrophage/neutrophil-specific IL-10 mutant animals (*IL-10*$^{FL/FL}$ *lysM-Cre*$^{+}$, D) responded with enhanced infiltration as well as extensive necrosis as in *IL-10*$^{-/-}$

mice. For comparison, untreated skin of WT mice is shown in E. Scale bars: right panels 200µm (x50), left panels 50µm (x400).

Figure 3. Survival and serum cytokine levels of various *IL-10* mutant mice and controls after systemic LPS exposure. A) Mice with cell type-specific inactivation of the *IL-10* gene in macrophages/neutrophils (*IL-10*$^{FL/FL}$ *lysM-Cre*$^{+}$, n = 10, □) or in T cells (*IL-10*$^{FL/FL}$ *CD4-Cre*$^{+}$, n = 10, Δ), mice with complete IL-10 deficiency (*IL-10*$^{-/-}$, n = 6, ■) as well as wt and *IL-10*$^{FL/FL}$ *Cre*$^{-}$ littermate controls (n = 25, ○) were injected with 20 µg of LPS/g body weight i.p..

B) Six *IL-10*$^{-/-}$, seven *IL-10*$^{FL/FL}$ *lysM-Cre*$^{+}$, nine *IL-10*$^{FL/FL}$ *CD4-Cre*$^{+}$ and seven *IL-10*$^{FL/FL}$ *Cre*$^{-}$ littermate controls were bled 6 hours after injection of 20 µg of LPS/g body weight i.p. and serum concentrations of TNF-α, IFN-γ and IL-12 were determined.

Figure 4. Local inflammatory response of *IL-10* mutant and WT mice to repetitive subcutaneous injection of CpG oligodeoxynucleotides. The CpG oligonucleotide (2.5 µg in 50 µl NaCl) was injected daily at the same site (flank) for five days. Tissue was sampled on day 6 and paraffin sections were stained with H&E or for chloroacetate esterase for convenient detection of neutrophilic granulocytes. Note the belt shaped inflammatory infiltrate (marked by vertical arrows) below the panniculus carnosus (muscle layer of murine skin, ✰) in WT mice (A and B). Vital keratinocytes of surface and follicular epithelium display abundant cytoplasm and large nuclei. The infiltrate is devoid of neutrophilic granulocytes (B). *IL-10*$^{-/-}$ animals (C and D) developed a more intense inflammatory infiltration with numerous neutrophilic granulocytes and extensive necrosis of epithelial structures as well as necrosis of dermis and panniculus carnosus (✰). Note that only shadows of

keratinocytes with condensed nuclei remain and that most of the epidermis is lost. The overview shows the margin of a large necrotic area extending over 3-5 medium power (x100) fields. Necrotic skin is marked by the horizontal arrow. T cell-specific *IL-10* mutant mice (*IL-10*$^{FL/FL}$ *CD4-Cre*$^{+}$, E and F) showed a similar response as observed in WT mice without tissue necrosis and without infiltration of neutrophilic granulocytes (F). Also the macrophage/neutrophil-specific *IL-10* mutant animals (*IL-10*$^{FL/FL}$ *lysM-Cre*$^{+}$, G and H) responded like WT animals. No tissue necrosis and no infiltrating neutrophilic granulocytes (H) were observed in these mice. For comparison, untreated skin of WT mice is shown (I and J). Scale bars: left and right panels 50µm (x400), centre panels 200µm (x50).

Figure 5. Serum cytokine levels in *IL-10* mutant mice and controls after daily subcutaneous injections of CpG oligonucleotides (2.5 µg in 50 µl NaCl) for 5 days. Serum was sampled on day 6. 'Mock' designates a negative control oligonucleotide without CpG motif.

Figure 6. IL-10 production by purified cell populations after stimulation *in vitro*. FACS-sorted splenic F4/80-positive macrophages and CD19-positive B cells were incubated in 96 well plates at a density of 2x10^5 cells/0.2 ml with either no stimulus or with a mock oligonucleotide, a CpG oligonucleotide or LPS. After 24 (open bars) or 48 hours (hatched bars), supernatant was harvested and assayed for IL-10 by ELISA.

Figure 7. Detection of cells carrying inactivated *IL-10* alleles in CpG-injected skin of macrophage/neutrophil-specific *IL-10* mutant mice by Southern blot analysis. After five successive daily subcutaneous injections of the CpG oligonucleotide in

two macrophage/neutrophil-specific *IL-10* mutant mice, lesional as well as untreated skin along with the superficial subcutaneous tissue was excised and DNA was extracted from the specimens. Southern blot analysis demonstrates an enhanced signal for the deleted *IL-10* allele (D) relative to the loxP-flanked (FL) allele in DNA from CpG-treated versus untreated skin.

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