# The Impact of TGF-ß, GM-CSF and Antibody Response for Diagnosis as well as Etiopathology of Lyme Disease

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***Summary***
In this study the pathogenesis of lyme disease was investigated *in vivo* by means of the wild-type mouse strains C3H/HeN and FVB/N as well as transgenic mouse strains, which are tissue specific modulated in their TGF-ß or GM-CSF (granulocyte-monocyte colony stimulating factor) activity and sensitivity. The mice were infected intradermally with *B. burgdorferi.* Progression of lyme disease was monitored using a number of diagnostic tests (recultivation of borrelia from tissue, ELISA, Western blot, histologic analysis). The study confirms the mouse strain FVB/N WT (wild type) to be susceptible to *B. burgdorferi* infections and that this represents a suitable *in vivo* model for investigations concerning the progression of a *B. burgdorferi* infection. We further demonstrate transgenic modification of TGF-ß or GM-CSF activity, and sensitivity in T cells and epithelium respectively, do not affect the pathogenesis of lyme disease. Our data show a positive linear correlation between antibody response and the severity of arthritic processes due to *B. burgdorferi* infection. Determination of the increase in antibody titer in sera of infected organisms therefore might be a useful tool to predict the progression of inflammatory processes in the course of lyme disease.

***Introduction***
Lyme disease is a chronic, inflammatory condition occurring in animals and men. Organs typically affected include the skin, joints, heart as well as the nervous system. The causative agents are a group of three closely-related, spiral bacteria species from the genus *Borrelia*: *Borrelia burgdorferi sensu stricto*, *Borrelia garinii and Borrelia afzelii (Steere, 2001*). All three species can be found in Europe. In the USA *B. burgdorferi* predominates (*Steere, 2001*).
The bacteria are transmitted during the blood meal of hard-shelled ticks. Not all infected individuals develop a clinically apparent disease. However, if a clinical manifestation occurs the disease can be classified into several phases. In the early stage (stage I), some days post infection, up to 60 % of the persons concerned develop a local infection of the skin, the so called erythema migrans (*Steere, 2001*). Weeks later (stage II) a neuroborreliosis emerges characterized by meningitis and neuropathy (*Steere, 2001*). In stage III, becoming manifest months or years post infection, the patients suffer from chronic inflammation of the heart (pericarditis), the joint (arthritis) or the nervous system (chronic neuroborreliosis) (*Steere, 2001*).
A number of parameters influence the severity of a lyme disease. The dose of spirochetes initially causing infection may be a pivotal point (*Yang et al., 1994*). In addition, various borrelia strains have been reported to differ in their infectivity (*Peavey and Lane, 1996*). Furthermore, lyme disease may be complicated by tick-borne co-infections e.g. babesiosis or human granulocytic anaplasmosis (*Thomas et al., 2001*). The immune response of the host is importance as well (*Barthold et al., 1990*).
So far, the mechanisms by which lyme disease is initiated and maintained are not well defined. In

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particular, it is not known why several patients develop a chronic lyme disease syndrome. Cytokines, however, are considered to play a pivotal role in the inflammatory processes linked to *B. burgdorferi* infection. For instance, the transforming growth factor-beta (TGF-ß) has been reported to be of importance in the pathogenesis of neuroborreliosis as well as lyme arthritis *(Montgomery et al., 2001, Widhe et al., 2002, Grygorczuk et al., 2003*). In addition, lyme disease has been described as being associated with granulocyte-monocyte colony stimulating factor (GM-CSF). Expression of GM-CSF is increased due to *B. burgdorferi* infection (*Pechova et al., 2002*). Moreover, the cytokine has been shown to promote a special way for spirochete internalization, the so called coiling phagocytosis *(Rittig et al., 1998*).

Diagnosis of lyme disease is hampered by the diversity of clinical signs. Direct and indirect detection methods as recultivation of borrelia from infected tissues as well as analysis of antibody response via ELISA or western blot technique are the common standard for verification of the disease. However, prediction of the prospective course of disease actually presents a problem. Thus, there is the need to elucidate new prognostic tools to assess the progression of inflammatory processes due to *B. burgdorferi* infection. In this study we approach this issue highlighting an interrelation between antibody response and inflammatory lesions. Furthermore, we elucidate the impact of TGF-ß and GM-CSF on the pathogenesis of lyme disease *in vivo* by mean of a murine infection model.

## *Materials and Methods*

### *Mice*

Generation and characterization of the transgenic mouse strains used TgN(CD2Tβ)1Mbl (TgTGF), TgN(CD2ΔkTβRII)2Mbl (TgdnRII), TgN(K5mGMCSF)2Mbl (TgGMCSF) and TgN(K10AGMCSF)2Mbl (TgAGMCSF) is described elsewhere *(Breuhahn et al., 2000, Mann et al., 2001, Schramm et al., 2003, Schramm et al., 2004*). The mouse strain TgTGF constitutively expresses biologically active TGF-β1 in T cells under the control of the human CD2 promoter. The mouse strain TgdnRII is T cell specific insensitive for TGF-β signaling caused by a truncated TGF-β type II receptor (ΔkTβRII) that is under the control of the human CD2 promoter. The mouse strain TgGMCSF constitutively overexpresses GM-CSF in the epidermis under the control of the bovine keratine 5 promotor. The mouse strain TgAGMCSF constitutively expresses a GM-CSF antagonist in the epidermis under the control of the bovine keratine 10 promotor. All transgenic mouse strains were established and maintained hemizygous on a FVB/N background. For investigations, female mice between 8 and 10 weeks of age of the transgenic mouse strains TgTGF, TgdnRII, TgGMCSF and TgAGMCSF as well as the wild type mouse strains C3H/HeN and FVB/N (both Charles River Laboratories International Inc., Wilmington, USA) were used. Mice were maintained in an individually ventilated caging system under environmental conditions of 21 °C, 50 to 60 % humidity, 20 air changes/h and a 12:12-hour light:dark cycle in accordance with the guidelines approved by the Animal Care and Usage Committee of the Regierungspräsidium Leipzig. Sterile food and water were given *ad libitum*. The mice were tested periodically for pathogens, in accordance with the recommendations for health monitoring of mice provided by the Federation of European Laboratory Animal Science Associations accreditation board. Trials were conducted in two independent experiments in groups of n = 7 for each mouse strain and time point investigated.

### *Cultivation of Borrelia burgdorferi sensu stricto N40 and infection of mice*

*B. burgdorferi sensu stricto* N40 were grown for ten days at 33 °C in modified Barbour-Stoenner-Kelly (BSK) medium as previously described (*Knauer et al., 2007*). Organisms were visualized by dark field microscopy and enumerated by using a Petroff-Hausser counting chamber. Anesthetized mice were infected using *B. burgdorferi sensu stricto* N40, passage 4 via injection of 100 µl BSK medium con-

taining $1 \times 10^7$ viable spirochetes intradermally into the shaved back.

*Isolation of B. burgdorferi from tissue samples*

Tissue samples from heart, brain, skin, tibiotarsal joint as well as bladder of infected mice were collected sterilely at 7, 14, 28 and 56 dpi (days post infection). Tissues were placed into 6 ml of modified BSK medium as previously described (*Straubinger et al., 1997*). The medium was incubated at 33 °C for six weeks, and was examined at 1-week intervals by dark-field microscopy for the presence of live spirochetes.

*Serology*

Serum samples were collected at 0, 7, 14, 28 and 56 dpi. Sera were tested for *B. burgdorferi* antibody levels by a kinetic enzyme-linked immunosorbent assay (KELA), and specific antibodies were detected by western blot analysis as described elsewhere *(Shin et al., 1993*).

**Table 1.** Isolation of *B. burgdorferi* from tissue samples

Mice of the transgenic mouse strains TgTGF, TgdnRII, TgGMCSF and TgAGMCSF as well as wild type mice (mouse strains C3H/He and FVB/N) were infected intradermally with *B. burgdorferi* organisms. Spirochetes were detected by culture in bladder, heart, brain, skin and tibiotarsal joint of infected mice 7, 14, 28 and 56 days post infection (dpi). n = 7 for each mouse strain and time point investigated.

**detection rate [%]**

| mouse strain | 7 dpi | 14 dpi | 28 dpi | 56 dpi |
|---|---|---|---|---|
| **C3H/HeN WT** | 71 | 86 | 100 | 100 |
| **FVB/N WT** | 100 | 100 | 100 | 100 |
| **TgTGF** | 71 | 100 | 100 | 100 |
| **TgdnRII** | 100 | 86 | 100 | 100 |
| **TgGMCSF** | 29 | 71 | 100 | 100 |
| **TgAGMCSF** | 86 | 100 | 100 | 100 |

**borrelia-positive tissues**

| mouse strain | 7 dpi | 14 dpi | 28 dpi | 56 dpi |
|---|---|---|---|---|
| **C3H/HeN WT** | bladder, heart, brain | heart, brain, skin, joint | bladder, heart, brain, skin, joint | bladder, heart, brain, skin, joint |
| **FVB/N WT** | bladder, heart, brain, skin, joint | bladder, heart, brain, skin, joint | bladder, heart, brain, skin, joint | bladder, heart, brain, skin, joint |
| **TgTGF** | bladder, heart, brain, joint | brain | bladder, heart, brain, skin, joint | bladder, heart, brain, skin, joint |
| **TgdnRII** | bladder, heart, brain, joint | bladder, heart, brain, joint | bladder, heart, brain, skin, joint | bladder, heart, brain, skin, joint |
| **TgGMCSF** | bladder, heart | heart, brain, skin, joint | bladder, heart, brain, skin, joint | bladder, heart, brain, skin, joint |
| **TgAGMCSF** | bladder, brain, skin, joint | bladder, heart, brain, skin, joint | bladder, heart, brain, skin, joint | bladder, heart, brain, skin, joint |

*Histologic analysis*

Tibiotarsal joints were removed from euthanized mice 0 dpi and 56 dpi, fixed in 10 % buffered formalin (VWR, Darmstadt, Germany), trimmed, embedded, cut and stained with hematoxylin and eosin (HE staining, VWR, Darmstadt, Germany) by standard procedures. Tissue slides were examined by the pathologist without knowledge of the protocol for each animal. The pathologic changes were classified using a histological score ranging from 1 to 6 with score 1 denoting no changes and score 6 denoting high-grade changes.

*Statistical analysis*

Data are shown as means ± standard deviation (SD). Two-way analysis of variance followed by unpaired Students *t* test was used to identify significant differences between means. Statistical analysis was carried out using the program GraphPad Prism 4 (GaphPad Software, La Jolla, USA). In all cases, $p < 0.05$ was assumed to indicate significant differences.

***Results***

Isolation of B. burgdorferi from tissues

*B. burgdorferi* organisms were isolated by culture from tissue samples of infected mice. The importance of the method is that it allows for the detection of viable, agile and

proliferating spirochetes. The recultivation of the borrelia organisms was conducted from heart, brain, skin, tibiotarsal joint as well as bladder. The bladder is a reservoir of the spirochetes and therefore is suited for verification of an infection. Heart, brain, skin and joints represent the organs particularly affected in the course of lyme borreliosis.

*B. burgdorferi* organisms could be detected in tissues of infected mice from all mouse strains tested (Tab. 1). Distinct differences in detection rates could be seen. Each mouse of strain FVB/N WT was positive per culture from each tissue even at 7 dpi. However, for mouse strain C3H/HeN WT a detection rate of 100 % was not obtained until 28 dpi. Moreover, overexpression of GM-CSF in the epithelium (mouse strain TgGMCSF) seems to repress the dissemination of the spirochetes at 7 dpi. However,

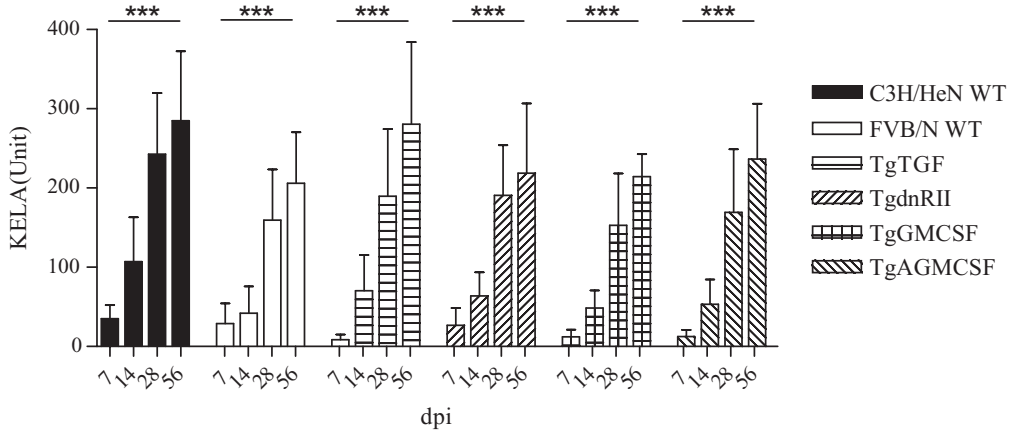


**Figure 1.** Quantitative antibody response to *B. burgdorferi* infection

Kinetic enzyme-linked immunosorbent assay (KELA) antibody units to *B. burgdorferi* in sera from mice of the transgenic mouse strains TgTGF, TgdnRII, TgGMCSF and TgAGMCSF as well as wild type mice (mouse strains C3H/He and FVB/N) 7, 14, 28 and 56 days post infection (dpi). Data are mean ± SD (n = 14; ***$p < 0.001$).

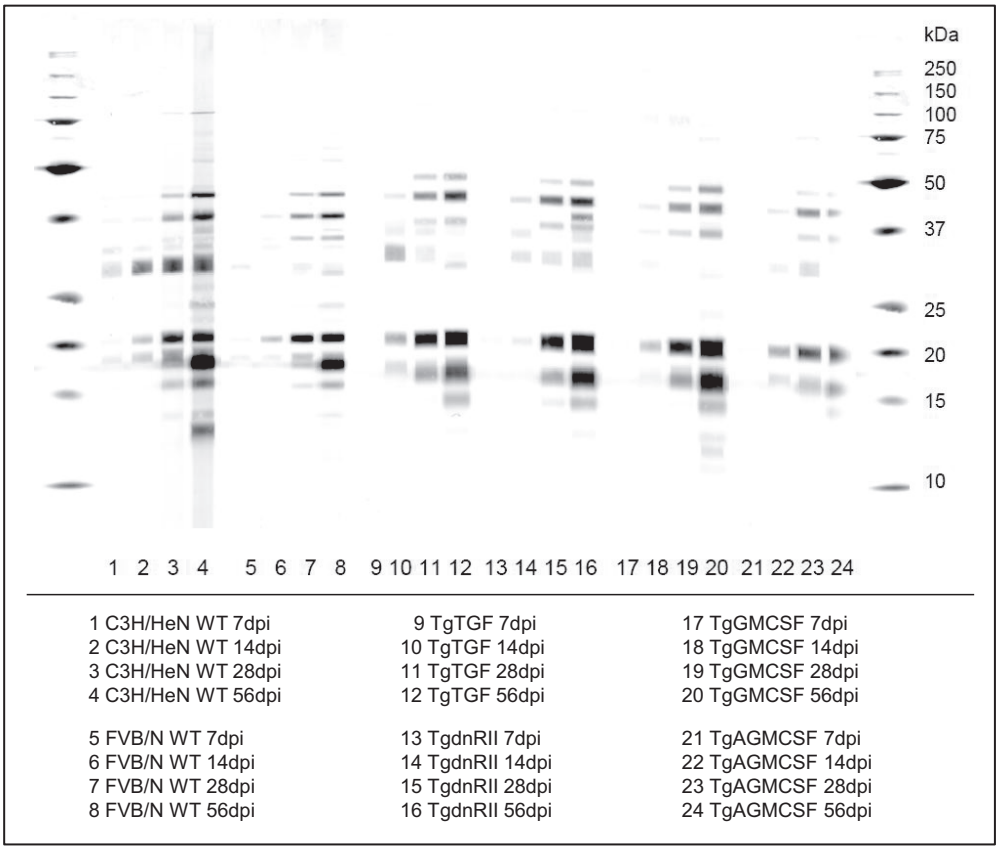


**Figure 2.** Qualitative antibody response to *B. burgdorferi* infection
Western blot analysis of sera from mice of the transgenic mouse strains TgTGF, TgdnRII, TgGMCSF and TgAGMCSF as well as wild type mice (mouse strains C3H/He and FVB/N) 7, 14, 28 and 56 days post infection (dpi). Data are representative for each mouse strain and time point investigated (n = 14).

from 28 dpi each mouse analyzed was positive per culture from each tissue regardless of its transgenic modifications. In addition, from this time point the borrelia organisms could be visualized in every tissue tested (heart, brain, skin, tibiotarsal joint, bladder) underlining the clinical manifestation of the infection. The spirochetes spread over the whole organism, affecting multiple tissues simultaneously.

*Serology*

Antibody response of infected mice was determined both quantitatively using a kinetic enzyme-linked immunosorbent assay as well as qualitatively by means of western blot analysis.

All mouse strains tested responded to the infection with *B. burgdorferi* and produced high antibody titers (200 – 300 KELA units) within 56 dpi (Fig. 1). The increase in antibody titer was particularly marked in the period from 14 to 28 dpi. Of note, the rise in antibody levels compares well between the transgenic mouse strains and the wild type. However, synthesis of specific antibodies was more pronounced for the mouse strain C3H/HeN WT compared to the mouse strain FVB/N WT characterized

by a significantly higher antibody titer in the serum of C3H/HeN WT (14 dpi: $p < 0.01$, 28 dpi: $p < 0.01$, 56 dpi: $p < 0.05$).

Changes in antibody titer were also reflected in western blot analysis. Infected mice of all mouse strains tested developed a specific pattern of bands typical for *B. burgdorferi* infection, which became more intense during the course of infection (Fig. 2). Specific bands were present at 14 kDa (p14), 17 kDa (Osp17), 19 kDa (OspE), 21 kDa (p21), 24 kDa (OspC), 30 kDa (p30), 34 kDa (OspB), 39 kDa (BmpA) as well as 43 kDa (p43). According to the results of the kinetic enzyme-linked immunosorbent assay, the antibody responses of the transgenic mouse strains were similar to the wild type. In addition, the antibody response of the mouse strain C3H/HeN WT was more intense than the antibody response of the mouse strain FVB/N WT. This became particularly noticeable for the protein signal in the range of 30 kDa.

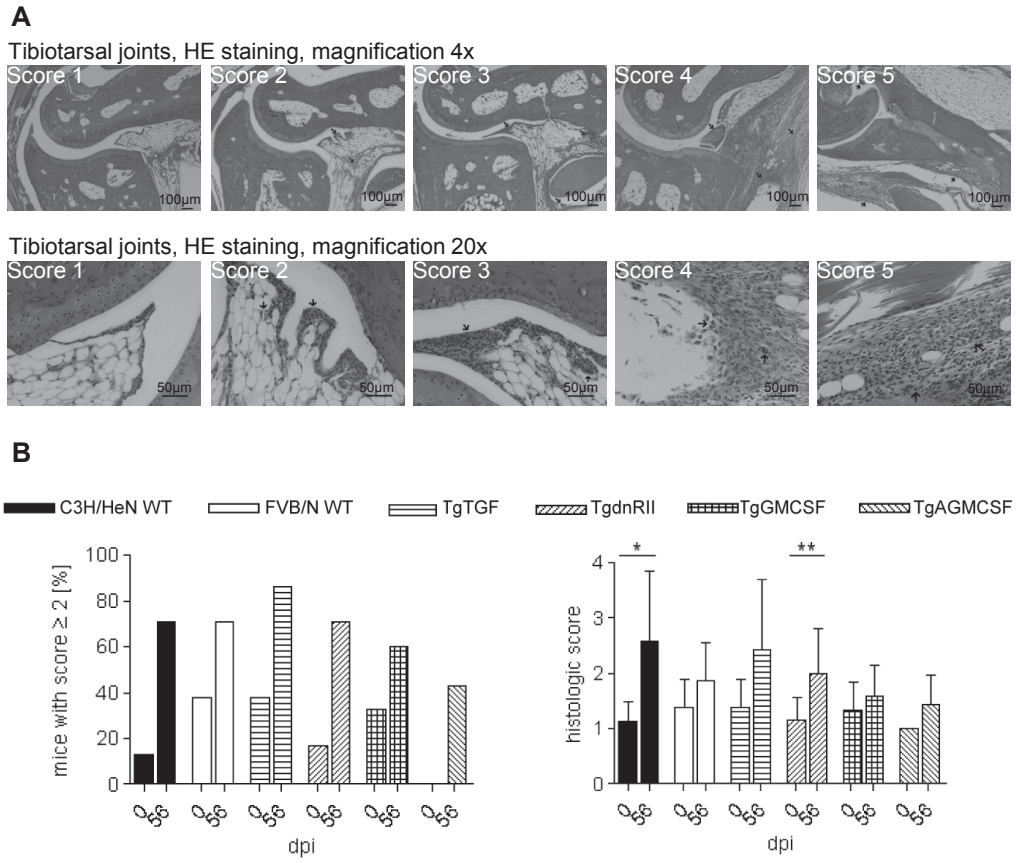


**Figure 3.** Histologic analysis

Tibiotarsal joints from mice of the transgenic mouse strains TgTGF, TgdnRII, TgGMCSF and TgAGMCSF as well as wild type mice (mouse strains C3H/He and FVB/N) were analyzed 0 and 56 days post infection (dpi). **A)** Hematoxylin eosin (HE) stained sections graduated according infiltrative as well as proliferative processes. **B)** Percentage of mice with inflammatory lesions (score ≥ 2) and histologic score identified for naïve as well as infected mice. Data are mean ± SD ($n = 7$; $*p < 0.05$).

*Histologic analysis*

The inflammatory lesions in the tibiotarsal joints of *B. burgdorferi* infected mice were characterized by focusing on infiltration of immune cells as well as proliferative processes of the synovial membrane. To quantify the pathologic changes a histologic score was used (Fig. 3 A).

Regardless of the transgenic modifications, due to infection with *B. burgdorferi,* an increasing number of mice with an inflammatory lesion (score ≥ 2) was observed (Fig. 3 B). The lesions were characterized by mononuclear cell infiltrations dominated by plasma cells below the synovial membrane reaching an intensity up to score 5. The increase in the histologic score of infected mice compared to naïve littermates was seen for all mouse strains tested, but was significant only for the mouse strains C3H/HeN WT and TgdnRII (Fig. 3 B).

*Interrelation of antibody response to inflammatory lesions*

A relation between antibody response and intensity of inflammatory lesions of infected mice could be seen (Fig. 4). Mice detected to have a greater increase in antibody titer were shown to reveal more severe pathologic abnormalities. The slope of regression line is significantly non-zero ($p < 0.01$). The regression line`s coefficient of determination is $R^2 = 0.8753$.

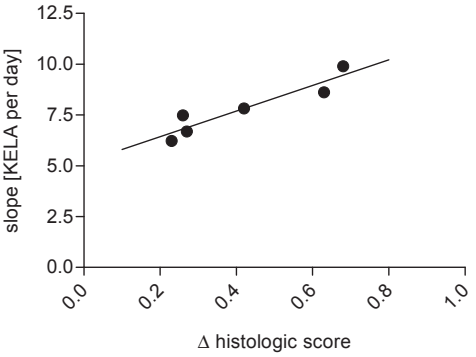


**Figure 4.** Interrelation of antibody response to inflammatory lesions

The difference in histologic score between naïve and infected littermates of the transgenic mouse strains TgTGF, TgdnRII, TgGMCSF and TgAGMCSF as well as wild type mice (mouse strains C3H/He and FVB/N) was plotted against the increase in antibody titer in sera of infected mice of the mentioned mouse strains in the period of 7 - 28 days post infection. Data are mean (n = 7). $R^2 = 0.8753$; $p < 0.01$

***Discussion***

Laboratory mice represent an appropriate model system for investigations concerning the pathogenesis of lyme disease. Both the clinical course and the histologic characteristics have been demonstrated to be similar between mice and men (*Barthold et al., 1990*). However, it should be noted that distinct mouse strains have been shown to differ in their susceptibility to develop chronic lyme disease (*Barthold et al., 1990*). Differences have been reported in rates of recultivation of borrelia as well as in antibody titer in sera of infected mice *(Barthold et al., 1990*). This is of special importance for validation of data from transgenic mice with different genetic backgrounds. So far, the mouse strains C3H/He, Balb/c and C57BL/6 have been approved as models for lyme disease in humans *(Barthold et al., 1990*). In this study we affirm the mouse strain FVB/N WT to be susceptible to infection with *B. burgdorferi*, too. The susceptibility of the mouse strain FVB/N WT for lyme disease could be verified both directly via isolation of *B. burgdorferi* from tissues of infected mice as well as indirectly by mean of immunological tests. Thus, our data confirm the mouse strain FVB/N WT to be a suitable *in vivo* model to investigate the pathogenesis of lyme disease.

Various studies report the cytokines TGF-ß as well as GM-CSF to be of importance in the pathogenesis of lyme disease. However, transgenic modification of TGF-ß or GM-CSF activity and sensitivity in T cells and epithelium respectively did not affect the susceptibility of mice in this study for development of lyme disease. Diagnosis of an occurring infection was achieved both via direct detection of the

causative agents and by means of immunological tests. For all mouse strains tested the spirochetes spread over the whole organism leading to a persistent infection. Moreover, regardless the transgenic modifications histologic analysis showed that mice developed inflammatory lesions in tibiotarsal joints due to *B. burgdorferi* infection. Nevertheless, at an early point of infection overexpression of GM-CSF in the epidermis (mouse strain TgGMCSF) seems to have a repressive effect on spirochete dissemination. GM-CSF is reported to activate antigen-specific granulocytes, monocytes, T cells and dendritic cells (*Cook et al., 2004*) and thus boost phagocytosis as well as production of superoxide radicals (*Lopez et al., 1986*). However, these mechanisms restricted to the epithelium are obviously not sufficient to impede development of lyme disease.

Numerous factors are considered to correlate with the severity of lyme disease. One important point linked to the intensity of inflammation is the antibody titer in sera of infected organisms. In fact, we could show a positive relationship between antibody response and inflammatory lesions. The coefficient of determination $R^2 = 0.8753$ and the p value $p < 0.01$ underline the power of the linear correlation. Accordingly, the slope in antibody titer represents a potent diagnostic marker to predict the progression of arthritic inflammatory processes due to lyme disease. Moreover, the determination of the decrease in antibody titer in sera of infected organisms may be a suitable tool for controlling the outcome of antibiotic treatment.

In summary, we report for the first time the mouse strain FVB/N WT to be susceptible to *B. burgdorferi* infections thus representing a suitable *in vivo* model for investigations concerning the progression of lyme disease. We further demonstrate transgenic modification of TGF-ß or GM-CSF activity, and sensitivity in T cells and epithelium respectively, do not affect the pathogenesis of lyme disease. Our data show a positive linear correlation between antibody response and the severity of arthritic processes due to *B. burgdorferi* infection. Determination of the increase in antibody titer in sera of infected organisms therefore might be a useful tool to predict the progression of inflammatory processes in the course of lyme disease.

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