Autoimmune skin inflammation is dependent on plasmacytoid dendritic cell activation by nucleic acids via TLR7 and TLR9

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Recognition of endogenous DNA and RNA by cells expressing TLR7 and TLR9 is an important contributor to the pathogenesis of systemic lupus erythematosus and has been suggested to contribute to cutaneous lupus and to a group of related inflammatory skin diseases termed interface dermatitis. We have developed a mouse model of TLR7– and TLR9–dependent skin inflammation using tape stripping. In normal mice, this resulted in a rapid but transient inflammatory cell infiltration accompanied by induction of type I IFN production by plasmacytoid dendritic cells (PDCs) and release of extracellular traps and proinflammatory cytokines by neutrophils. These responses were strongly reduced in MyD88–deficient mice and in mice treated with a bifunctional inhibitor of TLR7 and TLR9. In contrast, in lupus-prone (NZBxNZW)F1 mice, tape stripping induced the development of chronic lesions characterized by a persistent type I IFN gene signature and many clinical and histological features of cutaneous lupus. Depletion of PDCs before injury prevented the development of skin lesions, whereas treatment with a bifunctional TLR7/9 inhibitor before tape stripping or after the initial lesion was established led to a significant reduction of the disease. These data suggest that inhibitors of TLR7 and TLR9 signaling have potential therapeutic application for the treatment of interface dermatitis.

The triggering of TLR7 and TLR9 in plasmacytoid DC (PDC) precursors and B cells by self–nucleic acids is key in the pathogenesis of systemic lupus erythematosus (SLE). This leads to the production of type I IFNs from PDCs that can be detected by the up-regulation of IFN–regulated genes in the blood of patients with lupus (IFN signature) and anti-DNA and anti-RNP antibodies from B cells that form immune complexes (ICs) with DNA or RNA from dying cells (for reviews see Marshak-Rothstein, 2006; Barrat and Coffman, 2008). IFN-α and PDCs have been proposed to contribute to the pathogenesis of other autoimmune diseases characterized by IFN–α signature as well. Indeed, type I IFN–producing PDCs accumulate in the pancreas, muscle, and salivary glands of people affected by diabetes mellitus, dermatomyositis, and Sjögren’s syndrome, respectively, strongly suggesting that dysregulated PDC activation could be a more general feature of autoimmune disease (for reviews see Ueno et al., 2007; Barrat and Coffman, 2008; Guiducci et al., 2009).

PDCs and type I IFN appear to play a similar role in several cutaneous autoimmune diseases, including lichen planus, dermatomyositis, lichen sclerosis, cutaneous graft versus host disease and the cutaneous forms of lupus (cutaneous lupus erythematosus [CLE]; for review see Wenzel and Tüting, 2008). The common pathological feature of these diseases is interface dermatitis, a specific inflammatory pattern characterized by (a) vacuolar changes (liquefac-
by the absence of an animal model reflecting the central features of such diseases. In this study, we report development of a mouse model in which cutaneous injury by tape stripping leads to rapid infiltration and activation of PDCs and neutrophils. Although tape stripping causes a transient, self-limiting response in normal mice, the same treatment in a strain of lupus-prone mice produces a chronic lesion with many similarities to CLE. Our data thus suggest that when chronically activated, PDCs are a key player in inducing skin damage through sustained production of IFN-regulated genes as well as proinflammatory cytokines. Furthermore, we demonstrate that novel specific inhibitors of TLR7 and TLR9 can prevent skin damage when used in therapeutic settings.

RESULTS
Activated PDCs and neutrophils infiltrate skin rapidly after tape stripping
As a method to induce mild cutaneous injury and inflammation, we used tape stripping, a method previously used to provoke disease in mouse models of psoriasis and atopic dermatitis (Inoue et al., 2005; Sano et al., 2005; Jin et al., 2009). Tape stripping has also been used as a noninvasive method for detecting and diagnosing lupus, as lupus patients overreact to this mild cutaneous injury compared with healthy individuals. However, the nature of the inflammatory response to tape stripping has not been well characterized at the cellular or molecular level. At 24 h after tape...
stripping, we observed a pronounced increase of inflammatory cells in the skin (Fig. 1 B) compared with untreated skin (Fig. 1 A), including a cell population expressing the PDC markers CD11c+ and PDCA1+ (Fig. 1 B) as well as 120G8+ and Ly-6C (not depicted). The PDCs are functionally active as they produce IFN-α, measured by intracellular staining in cells isolated from the skin (Fig. 1 C). Flow cytometric analysis also revealed a massive influx of neutrophils (Ly-6G+ cells) and a lesser increase in macrophages (F4/80+ cells) along with CD4+ and CD8+ T cells (Fig. 1 B).

Activated neutrophils produce neutrophil extracellular traps (NETs), which are essential to kill bacteria in vivo (Brinkmann et al., 2004; Fuchs et al., 2007; for review see Wartha and Henriques-Normark, 2008). Neutrophils infiltrating tape-stripped skin are activated, producing abundant NETs with long chromatin fibers (Fig. 1 E), whereas no NET formation was observed in unstimulated BM neutrophils that we used as controls (Fig. 1 D). In skin neutrophils, the long fibers of the NETs contained both DNA and RNA and were associated with LL37/CRAMP (Fig. 1, F and G), a cationic antimicrobial peptide secreted by activated neutrophils (Kesenbrock et al., 2009; for review see Wartha and Henriques-Normark, 2008).

Signaling through TLR7 and TLR9 leads to rapid induction of IFN-regulated and proinflammatory genes at the site of cutaneous injury

Accompanying the cellular infiltration, epidermal injury resulted in strong induction of many prominent inflammatory genes in messenger RNA (mRNA) isolated from both skin biopsies and infiltrating leukocytes (Fig. 2 and Fig. S1). The induction of these genes required MyD88, as shown by the lack of gene induction in MyD88-deficient mice (Fig. 2 A and Fig. S1 A). To define the regulation of these genes, we repeated the experiment using IFNAR−/− mice that were lacking one chain of the type I IFN receptor. In these IFN-α/β–unresponsive mice, IFIT1, ISG15, IRF7, and ISG20, all IFN-regulated genes, were not induced (Fig. 2 B and Fig. S1 B) in either compartment, and IP-10 was reduced in the skin (Fig. 2 B) but not in the infiltrating cells (Fig. S1 B). Another IFN-regulated gene, IFI202, was induced in IFNAR−/− mice, which is consistent with previous studies showing an IFN-independent signaling pathway for this gene in mice (for reviews see Asefa et al., 2004; Choubey and Panchanathan, 2008). In contrast, the lack of IFN-α/β signaling did not reduce the induction of TNF, IL-1α, or IL-1β (Fig. 2 B and Fig. S1 B). Instead, expression of these inflammatory genes was somewhat increased in the skin (Fig. 2 B) but not in infiltrating cells (Fig. S1 B), possibly reflecting the previously reported reciprocal regulation of the type I IFN and TNF pathways (for review see Banchereau et al., 2004). The clear requirement for MyD88 in the induction of all three proinflammatory genes demonstrates an important role for signaling through members of IL-1 receptor or Toll-like receptor (TLR) families.

To test whether the two nucleic acid–specific TLRs, TLR7 and TLR9, were involved in the induction of these inflammatory genes signatures, we treated tape-stripped mice with IRS 954, a bifunctional oligonucleotide antagonist which blocks activation by TLR7 or TLR9 agonists in vitro (Barrat et al., 2005) and in vivo (Barrat et al., 2007). Treatment with IRS 954 significantly reduced the expression of both IFN–regulated genes and proinflammatory genes, in some cases reducing expression to the levels found in untreated mouse skin.
stripping in the individual receptor−deficient animals. We observed partial reduction of the levels of gene expression in the skin after injury in either TLR7 (Fig. S4 A)− or TLR9 (Fig. S4 B)−deficient mice. However, none of the genes were exclusively dependent on only one of these two receptors. As expected, the addition of the bifunctional TLR7/9 inhibitor IRS 954 completed the inhibition in the TLR9−deficient animals (Fig. S4 B).

PDCs and neutrophils are responsible for different patterns of cytokine induction in response to tape stripping

To determine the relative contribution of each of these two prominent cell types in this model of skin inflammation, we specifically depleted each cell type before tape stripping. Depletion of PDCs with the 120G8 monoclonal antibody led to strong reduction of the type I IFN−regulated genes (IFI202, IFIT, ISG15, ISG20, and IP-10) in infiltrating cells (Fig. S5) and skin biopsies (Fig. 4), whereas these genes were relatively unaffected by depletion of neutrophils (Fig. 4 and Fig. S5).

In contrast, neutrophil depletion resulted in a 70–90% reduction in TNF, IL-1α, and IL-1β mRNA, whereas depletion of PDCs led to a more modest 20–50% reduction in the expression of these genes. Concurrent depletion of both cell types, as expected, resulted in large reductions in the expression of both groups of genes (unpublished data). Collectively, these results suggest that in this acute skin injury model, PDCs and neutrophils are major components of the TLR7−, TLR9−, and MyD88−dependent inflammation but promote two separate inflammatory responses, one regulated by the type I IFNs produced by PDCs and one that involves neutrophil-dependent proinflammatory cytokines.

Lupus-prone (NZBxNZW)F1 mice develop chronic skin lesions resembling human CLE after tape stripping

Patients with SLE or CLE are often much more sensitive to mild cutaneous irritation and injury, suggesting that injury initiates a process that is exacerbated and sustained by autoimmune processes. Hybrid (NZBxNZW)F1 mice spontaneously develop high levels of circulating anti-DNA and RNA autoantibodies (for review see Furukawa and Yoshimasu, 2005), leading to IC formation and lupus nephritis resembling that observed in SLE patients. Although these mice rarely show spontaneous development of skin lesions, there is an accumulation of ICs at the epidermis−dermis junction, similar to those observed in human CLE (for reviews see McCauliffe, 1996; Furukawa and Yoshimasu, 2005). As ICs containing endogenous RNA or DNA are potent ligands for TLR7 and TLR9, respectively (Barrat et al., 2005; Means et al., 2005), we postulated that (NZBxNZW)F1 mice could exhibit a prolonged or exacerbated response to tape stripping.

The initial response to tape stripping in (NZBxNZW)F1 mice was very similar to that in normal mice, with involved skin showing a consistent increase in the number of PDCs and neutrophils (Fig. S6, A−C). The abundant cellular infiltrate was accompanied by increased expression of IFN−regulated and proinflammatory genes. A single injection of
IRS 954 before tape stripping efficiently inhibited this gene induction, which is consistent with a role of TLR 7 and TLR9 in driving the acute response in this strain as well (Fig. 6 D). However, in contrast to the transient course of inflammation in normal mice, the inflammatory response in (NZBxNZW)F1 mice was sustained for many days; mRNA for both IFN-regulated genes and proinflammatory cytokines remained significantly elevated up to 3 wk after tape stripping (Fig. 5 A). This suggests that either these mice fail to properly resolve the inflammation or conditions exist to perpetuate the inflammatory signals once initiated by the injury of tape stripping. 3 wk after tape stripping, skin lesions of (NZBxNZW)F1 mice extended over 50% of tape stripped area, whereas in normal mice, lesions were healed (Fig. 5, B and C). At very early time points, 1 and 4 d after tape stripping, (NZBxNZW)F1 mice had severe multifocal thinning of the epidermis with epidermal necrosis and diffuse dermoeipidermal leukocytic infiltrates composed largely of neutrophils and macrophages (unpublished data). By about 3 wk after the initial tape stripping, (NZBxNZW)F1 mice showed prominent epidermal hyperplasia with hyperkeratosis, keratin-filled craters or cysts, dermal fibrosclerosis, and degenerative changes of the s.c. fat tissue (Fig. 5, G–I and Fig. S7). In man, epidermal changes and vacuolar degeneration of the dermoepidermal junction are characteristic of all the forms of CLE, whereas other features of this model such as the presence of keratin-filled cysts and the degeneration of s.c. fat are more prominent in discreet CLE and verrucous discoid CLE (for review see Baltaci and Fritsch, 2009). Cutaneous lesions in (NZBxNZW)F1 mice showed persistent leukocytes infiltration, composed mainly of neutrophils, macrophages, and T cells, involving the epidermis, dermis, and adnexa as well as the s.c. fat (Fig. 5, G–I and Fig. S7). In the nonautoimmune mouse strains, 129 and 129 mice were either left untreated or tape stripped. PDCs and neutrophils were depleted before tape stripping using specific antibodies (250 µg given i.p. at day −2, day 0, and 8 h before tape stripping). Anti-120G8 antibody was used for depletion of PDCs and anti-GR1–Ly-6G antibody for depletion of neutrophils (Neut). Over 95% cellular depletion was achieved in both blood stream and skin infiltrate. 24 h after tape stripping, gene expression levels were measured in skin biopsies. Naive groups are shown for untreated mice only. Cumulative data from three independent experiments (n = 14 mice) are shown (mean ± SEM; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001).

PDCs and signaling through TLR7 and TLR9 are required for the initiation and maintenance of cutaneous lesions in (NZBxNZW)F1, mice

To demonstrate that PDCs and recognition of nucleic acids by TLR7 and TLR9 are central to the tape-stripping response in (NZBxNZW)F1 mice, animals were treated with IRS 954 before skin injury and for the duration of the experiment, according to the schedule in Fig. S8. In contrast to untreated mice (Fig. 6, A, D, and F), IRS 954–treated mice healed completely or had small lesions (<15% of the stripped area; Fig. 6, B and D). Skin from IRS 954–treated mice showed a very mild hyperplasia of the epithelium with slight hyperkeratosis and absence of ulceration. The inflammatory infiltrate and fibrotic reaction of the dermis appeared greatly reduced if compared with untreated animals, and no cellular infiltration was present in the epidermis (Fig. 6, G and H). Depletion of PDCs starting before tape stripping (Fig. S8) produced a very similar inhibition of the response to tape stripping. PDC-depleted mice had a normal appearance (Fig. 6, C and D) with normal to slight hyperplastic epithelium, negligible alterations of the dermis, epidermis, and adnexa, and minimal presence of inflammatory infiltration of the dermis (Fig. 6, I and J). Histological disease score confirmed these results and showed a significant difference between untreated mice and mice treated with IRS 954 or depleted of PDCs (Table 1). These results suggest that PDCs are key cells in the response to cutaneous injury through their ability to sense DNA and RNA through the two nucleic acid–specific TLRs expressed by PDCs.

To evaluate whether TLR7 and TLR9 signaling continues to be required for the prolonged response in (NZBxNZW)F1 mice or is primarily involved in the initiation of the response, we delayed the first IRS 954 treatment until 4 d after the tape.
and fibrosclerosis and negligible involvement of the adipose tissue (Fig. 7, F and G; and Table I). In summary, these findings demonstrate that the chronic activation of TLR7 and/or TLR9 in PDCs is required both to initiate and maintain the inflammation in the skin of (NZBxNZW)F1 mice. It also suggests that blocking this process with a specific TLR7/9 inhibitor such as IRS 954 is effective in a therapeutic setting, acting to resolve ongoing skin inflammation.

DISCUSSION

CLE belongs to a group of diseases named interface dermatitis (for review see Wenzel and Tüting, 2008) that shares a pattern of epidermal changes characterized by large areas of liquefaction at the derma–epidermal junction. The specific causes of these diseases are unknown, although the infiltration of IFN-α–producing PDCs in the area of vacuolization has been suggested to play a role in either initiating the disease and/or maintaining its chronicity (Farkas et al., 2001; for reviews see Wenzel and Tüting, 2007, 2008). The pathogenic effect of IFN-α in the skin could be explained in part by the induction of IFN-regulated cytokines such as IP-10, which induce the infiltration of pathogenic CXCR3+ T cells (for review see

stripping, at a time when the lesions were fully developed. Strikingly, the cutaneous lesions of mice treated with IRS 954 beginning at day 4 were almost completely healed by day 15–23 with only 10% of the surface still covered by open lesions (Fig. 7, B and C). In contrast, untreated animals had substantial unhealed lesions (Fig. 7, A and C–E) at these time points. Skin specimens from IRS 954–treated mice presented moderate alterations with very modest dermal inflammatory infiltration and fibrosclerosis and negligible involvement of the adipose tissue (Fig. 7, F and G; and Table I). In summary, these findings demonstrate that the chronic activation of TLR7 and/or TLR9 in PDCs is required both to initiate and maintain the inflammation in the skin of (NZBxNZW)F1 mice. It also suggests that blocking this process with a specific TLR7/9 inhibitor such as IRS 954 is effective in a therapeutic setting, acting to resolve ongoing skin inflammation.
Wenzel and Tüting, 2008). In addition, IFN-α has been shown to boost cytotoxic activity of T cells and to activate neutrophils to produce reactive oxygen intermediates, which mediate end-organ damage (Martinelli et al., 2004). Neutrophils that accumulate abundantly in the early phase of CLE lesions (for review see Lipsker and Saurat, 2008) also express both TLR7 and TLR9. Ligation of Fc-γ receptors by ICs activates these cells to produce proinflammatory cytokines, reactive oxygen intermediates, and proteases (Coxon et al., 2001; Hayashi et al., 2003; Tsuibo et al., 2008), thus playing an important role in the liquefaction process typical of CLE (Hayashi et al., 2003; for reviews see Lipsker and Saurat, 2008; Baltaci and Fritsch, 2009). Both type I IFNs and proinflammatory cytokines such as TNF, IL-1α, and IL-1β are abundantly expressed in CLE and can promote the migration of inflammatory cells to the skin (Popovic et al., 2005; for review see Werth, 2007).

Studies of the pathogenic mechanisms of CLE and development of new therapies for CLE have been hampered by the absence of animal models of interface dermatitis that replicate key features and pathways of the human disease (for review see Wenzel and Tüting, 2008). In this study, we report that skin injury caused by tape stripping in normal mice is followed by an acute inflammatory response accompanied by conspicuous infiltration of innate immune cells, including PDCs and neutrophils, paralleled with induction of type I IFN–regulated genes and proinflammatory cytokines. This increased expression of cytokine genes is completely abrogated in MyD88-deficient mice and in mice treated with a specific inhibitor of TLR7 and TLR9, indicating a central role for stimulation by RNA and/or DNA. Among the leukocytes that infiltrate the lesional skin, both PDCs and neutrophils express TLR7 and TLR9 receptors (Kadowaki et al., 2001; Edwards et al., 2003; Hayashi et al., 2003). The magnitude and composition of the cellular infiltrate are not significantly altered by TLR7 and TLR9 inhibition, suggesting that these pathways are not required for extravasation and homing. Using specific depleting antibodies, we demonstrate that PDCs, not neutrophils, are the source of type I IFN response, whereas proinflammatory cytokines IL-1α, IL-1β, and TNF are profoundly inhibited by neutrophil depletion. The reduction in cytokine gene expression in skin depleted of PDCs or neutrophils is similar in RNA samples extracted from infiltrating leukocytes or from whole skin biopsies, suggesting that keratinocytes or endothelial cells are not major contributors to this gene expression pattern. The finding that both IRS 954 and neutrophil depletion inhibit proinflammatory cytokines to a similar extent suggests that neutrophils do respond directly through one or both of these receptors. Alternatively, the inhibition of TLR7 and TLR9 may prevent induction of a factor made by another cell type (not PDCs) that activates neutrophils. The absence of TLR9 exacerbated disease in MRL/lpr lupus-prone mice, suggesting that TLR9 itself has a protective role in developing autoimmune inflammatory disorders (Christensen et al., 2006; Nickerson et al., 2010). However, we did not observe any exacerbation of skin inflammation in the TLR9-deficient animals.

The most likely ligands for TLR7 and TLR9 in injured skin are endogenous nucleic acids released from keratinocytes and other cell types dying as a consequence of mechanical injury or neutrophil cytotoxicity. Another potential source would be DNA specifically extruded from neutrophils in the form of NETs. This is not likely the major source of the initial TLR stimulation, as neutrophil depletion before injury does not significantly reduce the activation of PDCs (Fig. 4 and Fig. S5), however, this may be a relevant source in the chronic activation observed in autoimmune mice. Tissue injury in a largely sterile environment has been shown to stimulate a similar nucleic acid–dependent inflammatory response. Indeed, DNA release from necrotic hepatocytes stimulates cytokine production by neutrophils in a TLR9–dependent manner, and this has been suggested to be a primary mechanism of liver damage after some forms of hepatic injury (Imaeda et al., 2009; Bamboat et al., 2010).

In 129 or C57BL/6 mice, the burst of mRNA encoding inflammatory cytokines is transient, and gene expression levels return to pretreatment levels within 10 d of tape stripping. This is paralleled by reduction in cellular infiltration and progressive wound healing (unpublished data). Thus, this model represents an acute activation of pathways that are activated chronically in lupus, CLE, and related diseases. In contrast, tape stripping of lupus-prone (NZBxNZW)F1 mice produces a lesion quite similar to that in nonautoimmune

### Table I. Pathologic evaluation of skin lesions after tape stripping

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Epidermis thickness</th>
<th>Ulceration</th>
<th>Intraepithelial inflammation</th>
<th>Dermal inflammation</th>
<th>Panniculum inflammation</th>
<th>Total disease score</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NZBxNZW)F1 mice</td>
<td>2.9 ± 1.7</td>
<td>1.6 ± 0.2</td>
<td>1.0 ± 0.16</td>
<td>2.3 ± 0.14</td>
<td>1.7 ± 0.12</td>
<td>8.4 ± 0.6</td>
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<tr>
<td>C57BL/6 mice</td>
<td>0.4 ± 0.3</td>
<td>0.4 ± 0.4</td>
<td>0.4 ± 0.4</td>
<td>0.8 ± 0.2</td>
<td>1.4 ± 0.3</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>129 mice</td>
<td>1.0 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>1.6 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td>(NZBxNZW)F1 + IRS 954 (pre-injury)</td>
<td>0.7 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>0.8 ± 0.1</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>(NZBxNZW)F1 + IRS 954 (therapeutic)</td>
<td>1.0 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.2</td>
<td>1.2 ± 0.1</td>
<td>1.0 ± 0.0</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>(NZBxNZW)F1, depleted of PDCs</td>
<td>1.4 ± 0.3</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>4.0 ± 0.5</td>
</tr>
</tbody>
</table>

15–23 d after tape stripping, each of the histological features was scored singularly and graded 1 to 3 as indicated in Materials and methods; the overall disease score was calculated by combining the scores for each parameter. Data are from three to four different independent experiments (n = 12–30; mean ± SEM).

*P < 0.001 as compared with the (NZBxNZW)F1 group.

*P < 0.05 as compared with the (NZBxNZW)F1 group.
strains, but instead of healing spontaneously, it evolves into lesions that resemble the human CLE situation both clinically and histologically. The inflammation is mediated by TLR7 and TLR9 as the treatment of (NZBxNZW)F1 mice with the specific TLR7/9 inhibitor IRS 954 is able to prevent disease onset as shown by gross appearance of the skin, histological examination, and the normalization of the gene signatures. TLR7 and TLR9 activation is required not only for the induction of the inflammatory response but also for continuation of the chronic response seen in lupus-prone mice. This is shown clearly by the fact that IRS 954 treatment initiated after the development of the initial cellular infiltrate and cutaneous lesions leads to accelerated healing. This finding identifies TLR7 and TLR9 as important potential targets for therapy in CLE and related cutaneous autoimmune diseases.

Although the initial source of TLR ligands may be similar in normal and autoimmune mice, an important difference may be the continued presence of ligands for TLR7 and TLR9 in (NZBxNZW)F1 mice, specifically the ICs that accumulate at the dermis-epidermis junction (for reviews see McCauliffe, 1996; Furukawa and Yoshimasu, 2005) and the presence of circulating anti-DNA and RNA autoantibodies in these mice (for review see Furukawa and Yoshimasu, 2005). A similar phenomenon might take place in human CLE, where anti-DNA and RNA ICs in circulation and deposited in skin tissue have been extensively described (for reviews see McCauliffe, 1996; Wenzel and Tüting, 2008). Another source of DNA and RNA for this continued stimulation might be the neutrophils themselves. Skin neutrophils are highly activated after tape stripping and produce abundant NET fibers containing DNA and RNA molecules. NET-producing neutrophils were found in normal mice only at early time points, before the inflammatory response is resolved. However, in (NZBxNZW)F1 mice, significant infiltration of NET-producing neutrophils is detectable at later time points in well-established lesions (unpublished data), suggesting that they may constitute a source of endogenous nucleic acid leading to chronic TLR signaling. Interestingly, we found that the antimicrobial peptide LL37/CRAMP was associated with the fibers of the NETs from skin neutrophils. LL37/CRAMP is a cationic antimicrobial peptide highly inducible in keratinocytes and has been shown to convert endogenous DNA and RNA into a potent TLR9 or TLR7 agonist by promoting aggregation and enhanced uptake by PDCs in vitro (Lande et al., 2007; Ganguly et al., 2009). In addition to self-DNA/RNA, it is possible that microbial nucleic acids can participate in this process either directly by activating the TLRs or

![Figure 6](image-url). PDCs and TLR7 and TLR9 signaling are required for cutaneous disease formation in lupus-prone mice. (A–C) Macroscopical skin lesions 15–23 d after tape stripping in (NZBxNZW)F1 mice (A), (NZBxNZW)F1 treated with weekly injection of IRS 954 (B), and (NZBxNZW)F1, mice in which PDCs were depleted during the course of the experiment (C; see Fig. S8). (D) Quantification of area with open lesions 15–23 d after tape stripping in mice as in A–C. Cumulative data from at least two independent experiments (n = 12; mean ± SEM; *, P ≤ 0.05; **, P ≤ 0.01) are shown. (E–J) Representative sections of skin from untouched (NZBxNZW)F1, mice (naive; E) or from skin isolated 15–23 d after tape stripping from (NZBxNZW)F1 mice left untreated (F) or treated with IRS 954 (G and H) or depleted of PDCs (I and J). Representative sections from ~15 mice are shown. Bars, 200 µm.
indirectly by promoting the release of NETs by neutrophils. This is particularly relevant to the chronic model in which the skin is exposed for a long period of time.

The development of cutaneous lesions after injury in lupus-prone mice is similar to the Koebner phenomenon observed in patients with CLE and other autoimmune disease of the skin after skin trauma (for review see Ueki, 2005). The development of the lesions in (NZBxNZW)F1 is characterized by a persistent IFN signature and a high level of proinflammatory mediators such as IL-1α, IL-1β, and TNF similarly to the situation in human CLE (Werth et al., 2002; Clancy et al., 2004; Popovic et al., 2005; for review see Werth, 2007). The absence of spontaneous macroscopic lesions in lupus-prone mice is expected as most mouse model skin diseases require mild wounding (Spengel et al., 1999; Sano et al., 2005; Matsunaga et al., 2007).

In conclusion, we provide evidence that an abnormal response to endogenous ligands leading to chronic activation of both TLR7 and TLR9 may constitute a fundamental trigger of autoimmunity in the skin. The difference in outcome in lupus-prone versus normal mice after mild injury in the skin thus reflects the nature of the response, acute versus chronic, by PDCs and neutrophils to nucleic acids in the skin. Our data suggest that an abnormal/chronic response to TLR7 and TLR9 ligands can establish a self-perpetuating inflammatory loop driving diseases such as cutaneous lupus or other diseases with interface dermatitis. We also demonstrate that using novel oligonucleotide-based inhibitors of TLR7 and TLR9 could prove to be valuable therapeutics for skin autoimmune diseases.

MATERIALS AND METHODS

Reagents. Phosphorothioate oligodeoxynucleotides were prepared as previously described (Duramad et al., 2003). The prototype for inhibitor of TLR7 and TLR9 used was IRS 954, 5'-TGCTCCTGGAGGGTTGT-3' (Barrat et al., 2005). Control oligonucleotide was 5'-TCCTGACAGAT-3'. Mouse IFN-α ELISA sets were purchased from PBL Biomedical Laboratories.

Animals and in vivo treatments. C57BL/6 and 129 mice were purchased from Charles River. We purchased (NZBxNZW)F1 female mice from The Jackson Laboratory and used them at 18–22 wk of age. MyD88/KO and TLR9/KO mouse colonies were maintained at Simonsen Laboratories and were used with age-matched C57BL/6 WT controls at 8–12 wk of age. Animal experiments were conducted at MuriGenics. All animal experiments were approved by the institutional animal care and use committee of MuriGenics, in conformity with the Guide for the Care and Use of Laboratory Animals (National Research Council). We performed tape stripping after shaving the dorsal area (3 × 3 cm) using 10 strokes with duct tape. IRS 954 was administered just before tape stripping s.c. at a distant site. Alternatively, in (NZBxNZW)F1 mice, IRS 954 was administered long term, as depicted in Fig. S8. In certain experiments, PDCs and neutrophils were depleted with 250 µg IRS 954 given i.p. at day −2, day 0, and 8 h before tape stripping. We used anti-120G8 (Imgenex) for depletion of PDCs (Asselin-Paturel et al., 2003) and anti–GR1-Ly-6G (clone 1A8; BioLegend) for depletion of neutrophils (Daley et al., 2008). Over 95% cellular depletion was achieved in both blood stream and skin infiltrate. In experiments in which PDCs were depleted, long-term 120G8-depleting antibody was administered as depicted in Fig. S8. Experiments in (NZBxNZW)F1 mice were terminated between 15 and 23 d after initial tape stripping, depending on the progression of the lesions in the untreated groups in each experiment. The percentage of area with open lesions in (NZBxNZW)F1 and normal mice was evaluated with NIS-elements software (Nikon).

Histological analysis of skin inflammation and tissue pathology. The biopsy specimens were fixed in formalin and embedded in paraffin. Sections were stained with hematoxylin and
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concentration of 10^6/ml for 10 min at 37°C in RPMI and 2% FCS. After
view see Wartha and Henriques-Normark, 2008). In brief, skin-infiltrating
the last 2 h of stimulation. In some of the experiments, the ability of skin-
were stimulated for 4 h with CpG-C ISS; 5 µg/ml brefeldin A was added in
(PBL Biomedical Laboratories). As a positive control, BM-derived PDCs
lar infiltrate (see previous section) using an RNA micro kit (QIAGEN)
extracted from skin-infiltrating cells as described for the analysis of the cellu-
performed as described previously (Barrat et al., 2005). In brief, RNA was
DNA with SYTOX green (1:5000; Invitrogen; Fuchs et al., 2007). In some

Skin sample processing and flow cytometry. For analysis of cellular in-
filtrate, mice were killed 24 h later, and epidermis and dermis were mechani-
cally separated, followed by enzymatic digestion with 0.28 U/ml Liberase 3 (Roche) for 20 min at 37°C, passed through a 70-µm filter washed in RPMI
without serum, and counted. The cellular infiltrate was subsequently used for
preparation of RNA for TaqMan assay and/or stained for flow cytometric analyses. We performed flow cytometric analyses using fluorochrome-
conjugated monoclonal antibodies to mouse CD3, CD8, CD4, B220, CD11c
(tech), and 120G8 (Imgenex). Specific gating to characterize skin infiltrate
was performed as follows: PDCs were CD11c+, PDCA1^+; 120G8^+, Ly-6C^+,
myeloid DCs were CD11c^+ CD4^+; CD3^+CD8^+, neutrophils were GR1^–Ly-6G high F4/80
CD4^+; CD3^+CD8^–; neutrophils were GR1^–Ly-6G high F4/80^+; and macrophages
were GR1^–Ly-6G low F480^+. In the experiments in which IFN-α production by PDCs was evaluated by FACS analysis, skin was processed as
described for the analysis of the cellular infiltrate but in the presence of
5 µg/ml brefeldin A. Cellular infiltrate was seeded in noncoated plastic plates
at a concentration of 10^6/ml in RPMI medium (supplemented with 10% FCS) with the addition of 5 µg/ml brefeldin A for 2 h. Afterward, cells were
stained for surface markers with anti-CD11c plus anti-PDCA1-conjugated antibodies to identify PDCs. Cells were then fixed in 2% paraformaldehyde
and permeabilized for 10 min in 0.5% saponin and 1% BSA in PBS and then
stained in the same buffer with 5 µg/ml anti-IFN-α–conjugated antibody
(PBL Biomedical Laboratories). As a positive control, BM-derived PDCs
were stimulated for 4 h with CptG-C ISS; 5 µg/ml brefeldin A was added in the
last 2 h of stimulation. In some of the analyses, the ability of skin-
filtrating neutrophils to produce NETs was assayed as described previously (Brinkmann et al., 2004; Fuchs et al., 2007; Kessenbrock et al., 2009; for
review see Wartha and Henriques-Normark, 2009). In brief, skin-infiltrating
cells were seeded on coverslips (0.001% polylysine; Sigma-Aldrich) at a
concentration of 10^4/ml for 10 min at 37°C in RPMI and 2% FCS. After-
ward, cells were stained with anti-Ly-6G–conjugated antibody for 10 min on
ice and immediately fixed in 2% paraformaldehyde and counterstained for
DNA with SYTOX green (1:5000; Invitrogen; Fuchs et al., 2007). In some
experiments, after fixation, neutrophils were further stained with 5 µg/ml
anti-LL37/CRAMP antibody (Innovagen) and then counterstained for
DNA with SYTOX green (1:5000) or with a specific RNA dye SYTO RNA Select (1:5000; Invitrogen).

Real-time quantitative PCR (TaqMan) analysis. PCR reactions were
performed as described previously (Barrat et al., 2005). In brief, RNA was
extracted from skin-infiltrating cells as described for the analysis of the cellu-
lar infiltrate (see previous section) using a RNA micro kit (QIAGEN) and
from skin tissues with a fibrous tissue RNA extraction kit (QIAGEN)
according to the manufacturer’s instructions. RNA and cDNA were generated
with SuperScript First-Strand Synthesis System (Invitrogen). RT/PCR threshold
cycle (CT) values for each gene were normalized to the housekeeping gene
ubiquitin using the formula gene expression = 2^−(ΔΔCT) where the Ge and
Ct are the mean CT of duplicate runs of the gene of interest, and 100,000 is
arbitrarily chosen as a factor to bring all values above 1. Primers sequences
were used as follows: IFI202R, 5′-CTAGATGGCACCAGTCGT-TGG-3′; IFI202F, 5′-CAAGCTCCTGGACCA-TTA-3′; IFRT, 5′-TCCAAGCCTCCGGCCTAAGT-3′; IFRTF, 5′-ACAGGGCGTTT-TATCTTGGG-3′; ISG15R, 5′-CCCCCTTGTGTCTCACCAG-3′; ISGI5F, 5′-ACGGTCTACCTTTCACCAGT-3′; ISG20R, 5′-CCAC- CAGCTTGCTTCCTCGA-3′; ISG20F, 5′-GTACCGGCTTCAGCA-TATGTG-3′; NMIR, 5′-AATGGCTTTAATCCGGATCA-3′; NMIF, 5′-AGTTGAACGCGTTAGATGA-3′; IFIT1R, 5′-TCTGAGTATTA-ACCGCAGAC-3′; IFIT1F, 5′-AGGCTTGAGTTGCTGAGAT-3′; IL-1AR, 5′-CCGACAGCAGCGCTTTT-3′; IL-1AF, 5′-TGTCGTG- TGGACGTTCACCTT-3′; TNF-AR, 5′-GCTGCTGGGCATAGA- GAT-3′; TNF-ARF, 5′-GCCCACAGCCTTCTGTCTCT-3′; IL-1BR, 5′-AAAAGCTTTTTTCCCTTCTCTTCTT-3′; IL-1BF, 5′-GACGGC- ACACCCACCCCT-3′; IP-10F, 5′-GACGGTCGCTGCAACTG-3′; IP-10R, 5′-GACGGTGCGCTGCACTG-3′; IP-10R, 5′-GCTCCCTCATGGCCCTCATT-3′.

Statistical analysis. Data were analyzed using a two-tailed Student’s t test.
All analyses were performed using Prism software (GraphPad Software, Inc.).
Differences were considered significant at a P-value of <0.05.

Online supplemental material. Fig. S1 shows that the up-regulation of type 1 IFN–regulated genes in skin-infiltrating cells in normal mice is
IFNAR dependent. Fig. S2 shows that TLR7 and TLR9 inhibition does not
affect the ability of leukocytes to infiltrate the injured skin. Fig. S3 shows that stimulation of TLR7 and TLR9 is necessary for the up-regulation of type 1 IFN–regulated genes and inflammatory genes as measured in RNA samples from skin biopsies. Fig. S4 shows that the up-regulation of type 1 IFN–regulated genes and inflammatory genes is dependent on both TLR7 and TLR9 recep-
tors. Fig. S5 shows that the activation of both PDCs and neutrophils is critical for the burst of inflammatory genes after tape-stripping injury. Fig. S6 shows that in lupus-prone mice, (NZBxNZW)F1, tape stripping is followed
by up-regulation of type 1 IFN–regulated genes and inflammatory genes in a
TLR7– and TLR9–dependent way. Fig. S7 shows histopathological details in
lesions of (NZBxNZW)F1, at high magnification. Fig. S8 depicts the treat-
ment protocols in (NZBxNZW)F1 with the TLR7 and TLR9 inhibitor IRS
954 and in the PDC-depleting experiments. Online supplemental material is
available at http://www.jem.org/cgi/content/full/jem.20101048/DC1.

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Figure S1.  The MyD88 signaling pathway is necessary for the up-regulation of both type I IFN–regulated and proinflammatory genes in leukocytes infiltrating injured skin. (A) MyD88−/− (striped bars) and age-matched WT C57BL/6 mice (black bars) were either left untreated (naive) or tape stripped to provoke inflammation. 24 h later, infiltrating leukocytes were isolated, and the levels of proinflammatory genes were evaluated by TaqMan. (B) IFNAR−/− mice (striped bars) and age-matched WT 129 mice (black bars) were either left untreated (naive) or tape stripped to provoke inflammation. 24 h later, skin biopsies were isolated, and the levels of proinflammatory genes were evaluated by TaqMan. Naive (untreated) groups are shown for C57BL/6 and 129 WT mice only. Cumulative data from at least three independent experiments (n = 15–20) are shown (mean ± SEM; ** P ≤ 0.01; *** P ≤ 0.001).
Figure S2. TLR7 and TLR9 stimulation is not required for the cellular infiltration after skin stripping. 129 mice were left untreated (naive; white bars), tape stripped (TAPE; black bars), or tape stripped immediately after treatment (s.c.) with 100 μg of the dual TLR7 and TLR9 inhibitor IRS 954 as in Fig. 3. 24 h later, skin-infiltrating cells were isolated, and cell population was identified as in Fig. 1 B. Histograms show total cell number obtained from 2 x 2-cm skin biopsies (n = 10 mice) from two independent experiments (mean ± SEM).
Figure S3. Stimulation of TLR7 and TLR9 is required for the induction of skin inflammation. 129 WT mice were treated as described in Fig. 3. Genes were measured by TaqMan in skin biopsies. Cumulative data from at least two independent experiments (n = 10–15 mice) are shown (mean ± SEM; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001).
Figure S4. TLR7 and TLR9 signaling are both required for the induction of skin inflammation. (A) TLR7−/− (black bars) and age-matched WT C57BL/6 mice (gray bars) were either left untreated (naive) or tape stripped to provoke inflammation. 24 h later, skin biopsies were isolated, and the levels of proinflammatory were genes evaluated by TaqMan. (B) TLR9−/− were tape stripped (black bars) or tape stripped immediately after treatment (s.c.) with 100 μg of the dual TLR7 and TLR9 inhibitor IRS 954 (striped bars). Age-matched WT C57BL/6 mice were either left untreated (naive; white bars) or tape stripped (gray bars). 24 h later, skin biopsies were isolated, and the levels of proinflammatory genes were evaluated by TaqMan. (A and B) Cumulative data of two independent experiments are shown (n = 10 mice; mean ± SEM; *, P ≤ 0.05).

Figure S5. The activation of both PDCs and neutrophils is critical for the burst of inflammatory genes after tape-stripping injury. 129 mice were either left untreated or tape stripped. PDCs and/or neutrophils (Neut) were depleted before tape stripping using specific antibodies (250 μg given i.p. at day −2, day 0, and 8 h before tape stripping). Anti-120G8 antibody was used for depletion of PDCs, and anti-GR1–Ly-6G antibody was used for depletion of neutrophils. Over 95% cellular depletion was achieved in both blood stream and skin infiltrate. 24 h after tape stripping, gene expression levels were measured in skin-infiltrating cells. Naive groups are shown for untreated mice only. Cumulative data from three independent experiments (n = 14 mice) are shown (mean ± SEM; *, P ≤ 0.05; **, P ≤ 0.01).
Figure S6. Tape stripping of the skin of (NZBxNZW)F₁ mice provokes a robust inflammatory response that can be inhibited with IRS 954.

Characterization of the skin cellular infiltrate after inflammation via tape stripping in lupus-prone mice. (A–D) (NZBxNZW)F₁ were shaved and either left untreated (A) or tape stripped (B and C); 24 h later, skin-infiltrating cells were isolated (B and C), and gene expression levels (D) were evaluated by Taq-Man. Cells were characterized as described in Fig. 1 (A and B). (C) Histograms show total cell number obtained from 2 × 2-cm skin biopsies (n = 8 mice in one experiment; mean ± SEM). (D) (NZBxNZW)F₁ mice were left untreated (naive; white bars), tape stripped (TAPE; black bars), or tape stripped immediately after treatment with the dual TLR7 and TLR9 inhibitor IRS 954. 24 h later, gene expression levels were evaluated by TaqMan (n = 6 mice; in one experiment; mean ± SEM; *, P ≤ 0.05).
Figure S7. Details of histopathological features of lesions in (NZBxNZW)F1, 20 d after tape stripping. Representative sections of skin from (NZBxNZW)F1, 15–23 d after tape-stripping. (A–C) Hyperplasia and hyperkeratosis with fibrosclerosis and marked inflammatory infiltration of the dermis. (D) Alterations of the dermis–epidermis junction characterized by inflammatory infiltrate and degenerative modifications of the adipose tissue. (E and F) Details of infiltrate in the deeper dermis consisting of lymphocytes and abundant neutrophils (nuclear dust is indicated with an arrow in E and macrophages (boxed area in F). Representative sections from >20 mice are shown.
Figure S8.  Scheme for treatment protocols in (NZBxNZW)F1 with the TLR7 and TLR9 inhibitor IRS 954 and in the PDC-depleting experiments. IRS 954 was used starting the day of the tape stripping during the entire course of the experiments (Fig. 6, B, G, and H) or starting at 4 d after injury, when the disease was already established (Fig. 7, B, C, F, and G). In both cases, 100 μg IRS 954 (s.c.) was administered twice a week. Also described is the timing of PDC depletion experiments described in Fig. 6 (C, I, and J). 250 μg of depleting antibody to PDCs, anti-120G8 (i.p.), was given at day −2, day 0, and 8 h before tape stripping and then given twice a week for the entire duration of the experiment. Experiments were stopped in between 15 and 23 d after initial tape stripping depending on the progression of the lesions in the untreated groups.