Abrogation of macrophage migration inhibitory factor decreases West Nile virus lethality by limiting viral neuroinvasion

Alvaro Arjona, Harald G. Foellmer, Terrence Town, Lin Leng, Courtney McDonald, Tian Wang, Susan J. Wong, Ruth R. Montgomery, Erol Fikrig, and Richard Bucala

The flavivirus West Nile virus (WNV) is an emerging pathogen that causes life-threatening encephalitis in susceptible individuals. We investigated the role of the proinflammatory cytokine macrophage migration inhibitory factor (MIF), which is an upstream mediator of innate immunity, in WNV immunopathogenesis. We found that patients suffering from acute WNV infection presented with increased MIF levels in plasma and in cerebrospinal fluid. MIF expression also was induced in WNV-infected mice. Remarkably, abrogation of MIF action by 3 distinct approaches (antibody blockade, small molecule pharmacologic inhibition, and genetic deletion) rendered mice more resistant to WNV lethality. Mif−/− mice showed a reduced viral load and inflammatory response in the brain when compared with wild-type mice. Our results also indicate that MIF favors viral neuroinvasion by compromising the integrity of the blood-brain barrier. In conclusion, the data obtained from this study provide direct evidence for the involvement of MIF in viral pathogenesis and suggest that pharmacotherapeutic approaches targeting MIF may hold promise for the treatment of WNV encephalitis.

Introduction

Infection with West Nile virus (WNV) is an emerging health threat due to its ability to induce severe encephalitis that may lead to long-term neurological sequelae and death (1, 2). WNV is a mosquito-borne, single-stranded RNA flavivirus endemic to the Middle East, Europe, and Africa (3). It was introduced into North America in 1999 and is rapidly disseminating across the Western Hemisphere (4). Annual outbreaks of WNV fever and neuroinvasive disease occur in the United States (5), and no vaccines or specific therapies for WNV have yet been developed for humans.

The use of experimental animal models, especially genetically modified mice with defects in specific immune system molecules, has been providing insight into the immunopathogenesis of WNV infection. Several cytokines have been shown to play a significant role in the host response to WNV. For example, type I and type II interferons help control early viral replication and dissemination to the CNS (6, 7), and IL-1β is involved in WNV-induced Langerhans cell migration from the skin to the lymph nodes (8). In addition, we have previously reported that upon WNV infection, TLR3-mediated TNF-α production increases the permeability of the blood-brain barrier (BBB), thereby favoring WNV neuroinvasion (9).

Originally identified as a T cell cytokine (10), macrophage migration inhibitory factor (MIF) is currently considered a critical mediator of the inflammatory cascade and therefore the innate immune response (11). MIF is expressed in virtually all leukocytes (12) and is rapidly released from preformed, intracellular pools after challenge with bacterial products (11). Among other effects, MIF induces the production of inflammatory cytokines that in turn further stimulate MIF production, forming a positive feedback loop during the initial stages of the inflammatory cascade (13). Elevated levels of MIF are deleterious in sepsis and shock (14), and several reports have suggested involvement of MIF in viral pathogenesis. For example, among dengue virus–infected individuals, MIF serum levels were higher in patients showing hemorrhagic fever than in those presenting with the milder form of disease (15). Patients suffering from chronic HBV infection also presented with higher MIF serum levels than controls (16). In vitro studies have demonstrated that human CMV (HCMV) and influenza A virus infection induce MIF production (17, 18). Nevertheless, the involvement of MIF in viral infection remains, at best, largely uncharacterized. Our aim in this study was to investigate whether MIF influences WNV immunopathogenesis. We found that patients suffering from WNV infection presented with increased MIF levels in plasma and cerebrospinal fluid (CSF), and that abrogation of MIF action by genetic, immunoneutralization, or pharmacologic approaches protected mice from lethal WNV infection. MIF expression in mice favors viral neuroinvasion by compromising the integrity of the BBB. These data implicate MIF in the pathogenesis of lethal, neuroinvasive sequelae and suggest that pharmacologic inhibition of MIF may hold promise for the treatment of encephalitis due to WNV and possibly other flaviviruses.

Results

WNV infection induces MIF expression and protein production. MIF is rapidly released in response to microbial products (19, 20), and previous studies have shown that infection with several viruses such as Japanese encephalitis virus, HCMV, and HBV upregulates MIF expression (16, 18, 21). To assess whether WNV induces MIF, we determined MIF protein levels in patients suffering
from acute WNV infection. MIF plasma levels were significantly elevated in WNV patients when compared with controls (Figure 1A). Remarkably, we also found significantly elevated MIF protein levels in the CSF of WNV patients, while MIF was nearly undetectable in CSF samples obtained from uninfected donors (Figure 1B). Moreover, 2 patients that succumbed to WNV infection presented with extremely high MIF brain parenchymal levels (660 ± 212 ng/ml) that mirrored the CSF results. We also investigated the MIF response in an established model of WNV infection by analyzing MIF expression in WNV-inoculated mice over the course of infection. Figure 1C shows that MIF mRNA levels in the brain were significantly increased at day 6 postinfection (p.i.), when WNV starts to invade the CNS (9). Similarly, MIF mRNA levels in the spleen were also induced when WNV is first detected in this tissue (day 2 p.i.) and then declined steadily from day 4 to day 8 (Figure 1D), correlating with WNV peripheral clearance (22). A similar time course was observed in the MIF protein levels determined in the circulation of WNV-infected mice (Figure 1E). MIF levels in the blood of uninfected mice were barely detectable (data not shown). These data collectively indicate that MIF positively regulates MIF expression during the initial stages of infection and led us to further evaluate the involvement of MIF in WNV immunopathogenesis.

Figure 1

WNV infection induces MIF expression. (A) MIF plasma levels of patients suffering from acute WNV infection (n = 27) and control donors (n = 31). The distribution of data points in each group is represented by box-and-whisker plots, where horizontal lines mark the 25th, 50th (median), and 75th percentiles. The central vertical line in each box represents the range of values (5th–95th percentiles). (B) MIF levels in the CSF of WNV-infected patients (n = 7) and controls (n = 5). MIF mRNA levels were determined by Q-PCR in the brain (C) and spleen (D) of WT BALB/c mice at days 0, 2, 4, 6, and 8 after i.p. inoculation of WNV. Data are mean ± SEM of 3–6 mice per time point. (E) MIF serum levels determined by ELISA in WT BALB/c mice at days 0, 2, 4, 6, and 8 after i.p. inoculation of WNV. Data are mean ± SEM of 3–6 mice per time point. *P < 0.05, per Student’s t test. Data from mice were pooled from 2 independent experiments (3 mice per time point and experiment).

Reduced infection and inflammation in Mif<sup>−/−</sup> mouse brains. In the murine model of WNV infection, primary viremia and replication in peripheral tissues is followed by WNV crossing of the BBB and infecting the CNS at approximately day 5 p.i. (9). Analysis of WNV envelope protein (WNV-E) mRNA levels by quantitative PCR (Q-PCR) revealed that Mif<sup>−/−</sup> mice had a significantly reduced viral burden in the brain at days 6 and 8 p.i. when compared with WT mice (Figure 3A). Interestingly, the viral load in the spleen of Mif<sup>−/−</sup> mice was similar to that observed in WT mice at each of the time points studied (Figure 3B).

Confocal analysis of WNV-E antigen at day 6 p.i. revealed foci of infected cells in the olfactory bulb, cerebellum, and brain stem of WT mice, while the presence of infected cells was dramatically reduced in Mif<sup>−/−</sup> brains (Figure 4A). At this time point, we observed that WNV antigen is primarily found in meningeal areas, in accordance with previous reports (9). A similar pattern of results was observed with immunostaining for accumulation of leukocytes, as we found CD45-positive cells in the brain meninges of WT mice, but leukocytes were nearly absent in the corresponding regions of Mif<sup>−/−</sup> brains at day 6 p.i. (Figure 4A). Substantial WNV antigen and leukocyte infiltration were not found in Mif<sup>−/−</sup> mice until day 8 p.i. (Figure 4B), suggesting that MIF influences the kinetics of viral neuroinvasion. The brains of WT mice at day 8 p.i. nevertheless presented with increased levels of WNV antigen and leukocyte infiltration when compared with Mif<sup>−/−</sup> brains, particularly in the olfactory bulb (Figure 4B). These results are in accordance with the Q-PCR data, which showed that substantial levels of WNV-E mRNA in the brain of Mif<sup>−/−</sup> mice were not found until day 8 p.i., and that at this time point, brain viral burden in WT brains was approximately 14-fold higher (Figure 3A). Day 8 confocal analysis also revealed WNV infection not only of CD45-positive leukocytes but also of neurons, which resembles the tran-
sition from meningitis to meningoencephalitis described for this mouse model of WNV infection (9, 22).

We next measured cytokine mRNA levels in the brain tissue of WT and Mif–/– mice during the course of infection. In parallel to the time course of WNV neuroinvasion revealed by the Q-PCR and confocal analyses, the expression of the innate cytokines IFN-α, TNF-α, IL-6, and IL-12 was significantly increased in WT brains at days 6 and/or 8 p.i. The WNV-induced upregulation of inflammatory cytokines was significantly diminished in Mif–/– brains, and on average the mRNA levels for these cytokines were approximately 2- to 3-fold lower than those in WT brains at day 8 p.i. (Figure 5). These results are in line with the reduced leukocyte infiltration that was observed by immunohistochemistry (Figure 4).

Taken together, these data indicate that MIF contributes to WNV infection and the associated inflammatory reaction in the brain during the early phase of viral neuroinvasion (day 6 p.i.). By day 8 p.i., substantial brain viral burden and inflammation also were observed in Mif–/– mice, although to a lesser extent than in WT mice. This delay in neuroinvasion appears to be consistent with the survival data, as approximately 50% of the Mif–/– mice eventually succumbed to WNV infection (Figure 2).

**MIF facilitates WNV entry into the brain.** Previous studies have demonstrated that changes in the levels of peripheral cytokines that modulate the permeability of the BBB are responsible, at least in part, for the entry of viruses into the CNS (23–25). Because TNF-α receptor 1 signaling has been shown to regulate WNV neuroinvasiveness (9), and experimental data from multiple studies place MIF upstream of TNF-α in the inflammatory cascade (11), we examined peripheral TNF-α levels in WT and Mif–/– mice over the course of WNV infection. As shown in Figure 6A, splenic TNF-α mRNA levels were significantly increased at day 6 p.i. in WT animals. This WNV-induced upregulation of TNF-α was absent in Mif–/– mice. A similar trend was observed when we analyzed TNF-α protein levels in serum. Moreover, the TNF-α serum levels of WT mice were significantly higher than those of Mif–/– mice at day 6 p.i. (Figure 6B), coinciding with the time of viral neuroinvasion. A lack of WNV-induced cytokine upregulation in Mif–/– spleens also was observed in the case of IL-12 (Figure 6C), but not in the case of IL-1β (Figure 6D). In line with these observations, MIF immunoneutralization and pharmacologic inhibition have been previously shown to significantly reduce the production of inflammatory cytokines (14, 26). Indeed, we also found that serum TNF-α levels in ISO-1–treated mice (61.25 ± 2.72 pg/ml) were lower than those in the control group (90.25 ± 15.53 pg/ml) at day 6 after WNV infection.
To directly evaluate the role of MIF in BBB permeability, we injected poly(I:C)-challenged WT and \textit{Mif}^{-/-} mice with Evans blue, a dye that does not reach the CNS when the BBB is intact, using a previously characterized dose and a time course (9). Twenty-four hours after poly(I:C) challenge, we found evidence of BBB leakiness in WT but not in \textit{Mif}^{-/-} mice, as judged by macroscopic observation of Evans blue staining of the brain parenchyma. Importantly, \textit{Mif}^{-/-} mice also presented with reduced BBB permeability during WNV infection (Figure 7A). Finally, in order to further confirm that the reduced viral load observed in the brain and the enhanced survival of \textit{Mif}^{-/-} mice was due to decreased WNV neuroinvasion as opposed to some other mechanism, we equalized brain levels of the virus by directly injecting the same amount of WNV into the brains of WT and \textit{Mif}^{-/-} mice. We found no significant difference in survival between WT and \textit{Mif}^{-/-} mice when either 200 or 20 PFU of WNV was inoculated intracerebrally (Figure 7B, left and right panels, respectively). These data collectively support the conclusion that MIF promotes WNV invasion of the brain by compromising the integrity of the BBB.

**Discussion**

MIF is a potent proinflammatory cytokine with broad, upstream actions in the inflammatory cascade (20, 27). Once released, MIF exerts critical autocrine and paracrine activating effects, including the induction of inflammatory cytokines NO, COX-2, and prostaglandin E

![Figure 4](https://doi.org/10.1172/JCI32218)

Reduced infection and inflammatory cell infiltration in \textit{Mif}^{-/-} brains when compared with WT brains. Immunofluorescent confocal images from olfactory bulb, cerebellum, and brain stem double stained for WNV-E (WNV antigen) and CD45 at day 6 (A) and 8 (B) after i.p. inoculation of WNV. Original magnification, \texttimes25.
ing of the BBB (9), was significantly reduced in Mif<sup>−/−</sup> mice at the time of WNV entry into the brain. Third, Evans blue dye experiments revealed that Mif<sup>−/−</sup> mice have reduced BBB leakiness upon poly(I:C) or WNV challenge. Finally, we did not observe a difference in survival rate between WT and Mif<sup>−/−</sup> mice when WNV was directly inoculated into the brain (thus bypassing the BBB). Taken together, these data strongly suggest that MIF augments viral neuroinvasion by inducing the production of downstream inflammatory cytokines, chiefly TNF-α, that compromise the integrity of the BBB. Other mechanisms, such as MIF-mediated induction of matrix metalloproteinases (39, 40), which have been shown to regulate the integrity of the BBB (41), may also be involved in the MIF action on the BBB during WNV infection. These results also support our previous report showing that the stimulation of TLR3 signaling by WNV indirectly enhances the permeability of the BBB via the induction of TNF-α (9). Of note, we did not observe a difference between WT and Mif<sup>−/−</sup> mice when we analyzed their TLR3 mRNA levels in the spleen during WNV infection (data not shown), suggesting that the MIF-mediated TNF-α production in WNV-infected animals is independent of the TLR3 pathway.

While WNV infection in brain and the associated inflammatory responses were nearly absent in Mif<sup>−/−</sup> mice during the initial phase of neuroinvasion (day 6 p.i.), they were readily detectable at later time points (day 8 p.i.). Thus, the role of MIF in neuroinvasion is not essential, but contributory. The delay in lethal WNV neuroinvasion observed in Mif<sup>−/−</sup> mice nevertheless may allow for enhanced clearance of virus and improved survival. However, a substantial proportion of mice lacking MIF eventually develop WNV encephalitis, indicating that virus induction of inflammatory mediators important for breaching of the BBB can proceed in the absence of this cytokine.

It has been postulated that neurotropic viruses gain access to the CNS by direct entry into the brain through a permeabilized BBB or by the infiltration of infected leukocytes (9, 42). Bacher et al. (43) have suggested that the accumulation of MIF at the BBB may prevent macrophage infiltration after infection with Borre disease virus, and such action may be considered to agree with MIF’s eponymous “migration-inhibitory” activity (10). In contrast, we found that Mif<sup>−/−</sup> mice present a remarkably reduced number of infiltrating WNV-infected leukocytes in all the brain regions examined, particularly during the early stages of viral invasion. These results collectively indicate that: (a) the reduced levels of inflammatory cells and cytokines in Mif<sup>−/−</sup> brains are a consequence of a reduced viral neuroinvasion (and not vice versa); and (b) in the context of WNV infection, the proinflammatory action of MIF predominates over its capacity to inhibit leukocyte migration.

Increased serum MIF levels have been shown to positively correlate with disease severity and clinical outcome in patients infected with dengue virus (15). We found that patients suffering from acute WNV infection have significantly increased MIF levels in plasma and CSF. In our murine infection model, the increase in peripheral MIF levels is transient and coincides with the initial stages of viral infection (day 2). Similarly, increased MIF expression in the brain was found at the time of viral neuroinvasion (day 6) but rapidly declined. Even the transient increase in MIF peripheral levels appeared sufficient to critically shape the subsequent host immune response, as TNF-α and IL-12 peripheral levels were significantly reduced at day 6 p.i. in Mif<sup>−/−</sup> animals. These results further support the placement of MIF upstream of the host inflammatory reaction that results from pathogen invasion (14, 20).

**Figure 5**
Mif<sup>−/−</sup> mice have reduced levels of inflammatory cytokines in the brain in response to WNV infection. IFN-α (A), TNF-α (B), IL-6 (C), and IL-12 (D) levels were determined by Q-PCR in the brain of WT and Mif<sup>−/−</sup> mice at days 4, 6, and 8 after i.p. inoculation of WNV. Data are mean ± SEM of 6–8 mice, pooled from 2 independent experiments. *P < 0.05, compared with day 4 per Student’s t test; †P < 0.05, compared with WT group per 2-way ANOVA.

**Figure 6**
Peripheral cytokine response to WNV infection in WT and Mif<sup>−/−</sup> mice. TNF-α mRNA levels in the spleen (A) and protein levels in serum (B), IL-12 mRNA levels (C), and IL-1β mRNA levels (D) in the spleen were determined at days 2, 4, and 6 after i.p. inoculation of WNV. Data are mean ± SEM of 6–8 mice per group per time point obtained from 2 independent experiments. *P < 0.05, compared with day 2 per Student’s t test; †P < 0.05, compared with WT group per 2-way ANOVA.
The data presented herein demonstrate that MIF plays a pivotal role in the immune response to WNV infection and provide direct evidence for the involvement of MIF in viral pathogenesis. WNV has been rapidly spreading in the Western Hemisphere since 1999, causing at least 3,830 cases and 119 fatalities (44). No specific or proven treatment is currently available. Remarkably, we show that abrogation of MIF by 3 different approaches (antibody blockade, pharmacologic inhibition, and genetic deletion) rendered mice more resistant to lethal WNV infection. MIF pharmacologic inhibition has been proven to be feasible and efficacious in several experimental models of inflammation and shock (45–47), and our study opens a promising avenue for the development of MIF-blocking therapies for WNV infection and possible other viral encephalitides. Indeed, MIF inhibition has been found to protect from bacteria-induced shock in the same experimental infection models in which inhibition of downstream inflammatory factors such as TNF-α showed no effect (14). In addition, studies on MIF gene polymorphisms have revealed the existence of high-expression MIF alleles that are linked to increased susceptibility and severity of various inflammatory diseases (48–51). Investigating whether MIF polymorphisms influence the susceptibility to WNV encephalitis may be valuable for therapeutic and prognostic purposes as well as for assessing which individuals may benefit most from potential MIF-directed pharmacotherapies.

Methods
Human samples. Plasma and CSF samples (n = 27 and n = 7, respectively) from patients suffering from acute WNV infection (ages 15–77 years, mean 54.5, SD 22.6) and from disease-free donors (ages 17–53 years, mean 31.5, SD 11.4) asymptomatic for viral infection were provided by the Diagnostic Immunology Laboratory of the Wadsworth Center (New York State Department of Health). We did not observe any correlation between age and plasma MIF levels, which is in accordance with prior publications on MIF levels in acute disease (52). Serodiagnosis of WNV was based upon the results of WNV ELISA-IgM followed by neutralization tests on paired sera. The majority of the patients had symptoms of encephalopathy that were attributed to WNV infection, including the 2 patients who presented with high brain parenchyma MIF levels. Samples were stored at −70°C until analyzed for MIF levels as described below. Samples were obtained following protocols approved by New York State and Yale Institutional Review Boards, and an informed consent was obtained from participants.

Figure 7
MIF facilitates WNV entry into the brain. (A) BBB permeability is increased after 24 hours of poly(I:C) challenge or 4 days after WNV infection in WT but not in Mif−/− mice. Representative photographs of Evans blue dye staining of whole brains. Original magnification, ×0.5. (B) Survival analysis did not reveal a statistically significant difference (P > 0.1) between WT and Mif−/− mice after intracerebral inoculation of 200 PFU (left panel) or 20 PFU (right panel) of WNV (n = 10 mice per group).

Mice. Mice were obtained from Taconic or were bred at the Yale Animal Resources Center under pathogen-free conditions. We used female Mif−/− mice, 6 weeks of age, backcrossed into the BALB/c genetic background (generation N10) (49), and age-matched, female BALB/c mice as controls. Experimental protocols followed Yale University institutional guidelines for care and use of laboratory animals, and were approved by the Institutional Animal Care and Use Committee (IACUC) at Yale University.

Reagents. A previously described monoclonal anti-MIF antibody of IgG1 isotype (NIHXIV15.5) neutralizes mouse MIF bioactivity and has been shown to protect from lethal, bacterial infection (14, 29). The inhibitor ISO-1, which binds to the N-terminal region of MIF and inhibits its proinflammatory activity (46), was purchased from Calbiochem (EMD Biosciences).

WNV infection and treatments. We inoculated mice i.p. with of WNV isolate 2741 (100 PFU per mouse) in 200 μl of PBS containing 1% gelatin (7). For blocking experiments, mice were injected i.p. with 500 μg of anti-MIF mAb or control IgG1 on days 1, 3, and 6 p.i. For pharmacologic inhibition of MIF, we injected mice i.p. with 1 mg of ISO-1 or vehicle (H2O/10% DMSO; total volume 200 μl) 1 day before WNV inoculation and then daily from days 1 to 7 p.i. Intracerebral inoculation of WNV was performed as described previously (9). Animals were monitored daily for WNV-associate symptoms and mortality.

Cytokine ELISA. MIF concentrations in plasma were measured by sandwich ELISA using human- or mouse-specific antibodies prepared in our laboratory (14). Mouse serum TNF-α levels were determined using a commercial ELISA (R&D Systems) following the manufacturer’s instructions.

Q-PCR analysis of viral load and cytokines. We extracted total RNA from frozen tissue samples using the RNeasy extraction kit (QIAGEN), and cDNA was synthesized from 1 μg of RNA using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was carried out with the iQ SYBR Green system (Bio-Rad) using previously published primers for WNV-E (9), IFN-α, TNF-α, IL-6, IL-12p40, IL-1β (53), and MIF (54). Emitted fluorescence for each reaction was measured during the annealing/extension phase, and relative quantity values were calculated by the standard curve method. We used the quantity value of actin in each sample as a normalizing control.

Immunofluorescence and confocal microscopy. Mice were sacrificed and perfused transcardially with ice-cold PBS as described previously (9). Brains were fixed in 4% paraformaldehyde overnight at 4°C, followed by cryoprotection with 10% (wt/vol), 20% (wt/vol), and 30% (wt/vol) sucrose in PBS for 24 hours each before embedding in optimal cutting temperature compound. Subsequently, specimens were processed, and histological slides were prepared for staining with rat antibody specific for mouse CD45 (clone YW 62.3; Serotec) and biotinylated mouse ascites fluid WNV antibody (used at 1:200 and 1:500, respectively, and incubated for 16 hours at 4°C; ref. 9). We incubated frozen sections overnight at 4°C with antibodies, washed and incubated the sections with secondary reagents including goat anti-rat IgG conjugated with Alexa Fluor 568 (Molecular Probes; Invitrogen) and streptavidin conjugated with Alexa Fluor 488 (Molecular Probes; Invitrogen), each used at 1:200 and incubated for 1 hour at ambient temperature. We mounted sections with ProLong Gold fluorescence mounting medium containing DAPI (Invitrogen). Immunofluorescence
was observed by confocal microscopy in independent channels using a Zeiss LSM 510META microscope (Carl Zeiss Microimaging).

Evaluation of BBB permeability. We challenged mice i.p. with 50 μg of poly(I:C) or with 100 PFU of WNV. Twenty-four-hours (poly(I:C)) or 4 days (WNV) later, mice were injected with 800 μl of 1% (w/v)ol Evans blue dye, and after 1 hour brains were excised. These doses and time points have been optimized by us previously (9).

Statistics. We analyzed differences between means using the unpaired 2-tailed Student’s t test or ANOVA. To assess statistical differences between survival rates, we employed the $\chi^2$ test. A P value less than 0.05 was considered significant.

Acknowledgments

This work was supported by awards from the NIH (AI50031, AI055749, AI42310). T. Town is supported by an Alzheimer's Association grant and an NIH/National Institute on Aging “Pathway to Independence” award (1K99AG029726).

Received for publication March 26, 2007, and accepted in revised form July 11, 2007.

Address correspondence to: Erol Fikrig, Section of Rheumatology, Department of Internal Medicine, Yale University School of Medicine, PO Box 208031, New Haven, Connecticut 06520-8031, USA. Phone: (203) 785-2453; Fax: (203) 785-7053; E-mail: erol.fikrig@yale.edu.

Tian Wang's present address is: Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, Colorado, USA.