Interleukin 1β Blockade Reduces Intestinal Inflammation in a Murine Model of Tumor Necrosis Factor–Independent Ulcerative Colitis

Marina Liso,1 Giulio Verna,2,3 Elisabetta Cavalcanti,1 Stefania De Santis,4 Raffaele Armentano,1 Angela Tafaro,1 Antonio Lippolis,1 Pietro Campiglia,2 Antonio Gasbarrini,5 Mauro Mastronardi,1 Theresa Torres Pizarro,3,6 Fabio Cominelli,3,6 Loris Riccardo Lopetuso,5,7,8,§ and Marcello Chieppa1,9,10,§

1National Institute of Gastroenterology “S. de Bellis,” Research Hospital, Castellana Grotte (BA), Italy; 2Department of Pharmacy, University of Salerno, Fisciano (SA), Italy; 3Digestive Health Research Institute, Case Western Reserve University School of Medicine, Cleveland, Ohio, USA; 4Department of Pharmacy-Drug Science, University of Bari Aldo Moro, Bari, Italy; 5Digestive Disease Center–Unità Operativa Complessa di Medicina Interna e Gastroenterologia, Dipartimento di Scienze Mediche e Chirurgiche, Fondazione Policlinico Universitario “A. Gemelli” Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Roma, Italy; 6Department of Pathology, Case Western Reserve University School of Medicine, Cleveland, Ohio, USA; 7Department of Medicine and Ageing Sciences, “G. d’Annunzio” University of Chieti-Pescara, Chieti, Italy; 8Center for Advanced Studies and Technology, “G. d’Annunzio” University of Chieti-Pescara, Chieti, Italy; 9Dietetics and Clinical Nutrition Laboratory, Department of Public Health, Experimental and Forensic Medicine, University of Pavia, Pavia, Italy; and 10Department of Biological and Environmental Sciences and Technologies (DiSTeBA), University of Salento, via Monteroni, Lecce, Italy

SUMMARY
Anakinra administration represents a therapeutic option for tumor necrosis factor–independent ulcerative colitis subjects. Circulating interleukin 1β can predict the candidate subpopulation of ulcerative colitis patients non responder to the anti-tumor necrosis factor therapy, who may benefit from anakinra administration.

BACKGROUND & AIMS: Inflammatory bowel diseases are multifactorial diseases commonly treated with either immunomodulatory drugs or anti–tumor necrosis factor (TNF). Currently, failure to respond to anti-TNF therapy (assessed no earlier than 8–12 weeks after starting treatment) occurs in 20%–40% of patients enrolled in clinical trials and in 10%–20% in clinical practice. Murine models of inflammatory bowel disease provide important tools to better understand disease mechanism(s). In this context and among the numerous models available, Winnie–TNF–knockout (KO) mice recently were reported to show characteristics of ulcerative colitis (UC) that are independent of TNF, and with increased interleukin (IL)1β production.

METHODS: Herein, the efficacy of recombinant IL1-receptor antagonist (anakinra) administration was evaluated in Winnie-TNF-KO mice, used as a UC model of primary anti-TNF nonresponders.

RESULTS: We analyzed gut mucosal biopsy specimens and circulating cytokine profiles of a cohort of 30 UC patients;
approximately 75% of primary nonresponders were characterized by abundant IL1β in both the serum and local intestinal tissues. In Winnie-TNF-KO mice, administration of anakinra efficiently reduced the histologic score of the distal colon, which represents the most common site of inflammation in Winnie mice. Furthermore, among lamina propria and mesenteric lymph node–derived T cells, interferon-γ–expressing CD8+ T cells were reduced significantly after anakinra administration.

CONCLUSIONS: Our study provides new insight and alternative approaches to treat UC patients, and points to anti-IL1 strategies (ie, anakinra) that may be a more effective therapeutic option for primary nonresponders to anti-TNF therapy. (Cell Mol Gastroenterol Hepatol 2022;14:151–171; https://doi.org/10.1016/j.jcmgh.2022.03.003)

Keywords: Ulcerative Colitis; Cytokines; TNF.

Inflammatory bowel diseases (IBDs) are gastrointestinal disorders characterized by dysregulated immune responses and prominent dysbiosis, which in turn leads to chronic relapsing inflammation of the gut. Environmental factors, as well as a genetic predisposition, also are important contributors to disease pathogenesis. IBDs include ulcerative colitis (UC), which manifests as inflammation restricted to the mucosal surface of the colon, and Crohn’s disease, which is more transmural in nature and can affect the entire length of the gastrointestinal tract. For both, one main immunopathogenic cause is the dysregulated equilibrium between proinflammatory and regulatory cytokines. Several cytokines have been reported to play a pivotal role in the activation of innate and adaptive immune responses, of which tumor necrosis factor (TNF) has been found in the sera, colonic mucosa, and stool of UC patients.1–3 The development of anti-TNF biologics with the ability to block this key proinflammatory cytokine represents a significant improvement in the quality of life for IBD patients, reducing the need for surgical interventions and improving, and often inducing, persistent clinical remission.4 Nonetheless, variability of disease involvement, as well as response to therapy, is a commonplace occurrence for patients with IBD.5

Anti-TNF agents are not a magic bullet for all IBD cases. First, side effects can be severe and life-threatening in some patients, mainly owing to infective events and then to immunogenicity, with the formation of antibodies to anti-TNF and consequent loss of response to these drugs over time. In fact, primary nonresponse to anti-TNF induction therapy occurs in 20%–40% of patients in a clinical trial setting. Secondary loss of response is also a common clinical problem, with the incidence ranging between 23% and 46% at 12 months after anti-TNF initiation.6–8 Using a cohort of 955 patients treated with infliximab (IFX), Kennedy et al9 reported a 23.8% incidence of primary nonresponse. A suboptimal drug concentration at week 14 was associated with primary nonresponse, but this could be only partially explained by the development of antidrug antibodies.9–11 In these cases, the clinical management of this second group of patients implies the use of alternative drugs blocking different molecules with different rates of success.

In a recent study, interleukin (IL)1β was identified as one of the most important biomarkers associated with the failure of anti-TNF therapy.12 IL1β is produced by limited types of cells, including macrophages, dendritic cells (DCs), and monocytes, and requires a series of intracellular events to occur before becoming completely active and ready to trigger inflammation.13 The IL1 axis, which includes IL1α and IL1β binding to the same receptor (IL1R1: Interleukin 1 Receptor Type 1), has been identified repeatedly as a crucial mediator for the onset of UC inflammation.14–16 IL1-receptor antagonist (IL1Ra) can inhibit the biological effects of IL1, acting as a natural decoy for IL1β.16 Anakinra (Kineret, Swedish Orphan Biovitrum AB, Stockholm, Sweden), a recombinant form of IL1Ra, is able to inhibit the effects of IL1β17 and has been used successfully for the treatment of patients with rheumatoid arthritis18–20 and severe sepsis.21

Recently, the association of IL1β with primary nonresponse to anti-TNF was shown.22,23 In the present study, we analyzed circulating cytokine profiles and intestinal biopsy specimens of a cohort of UC patients. Significant differences in IL1β were associated with primary nonresponse to anti-TNF administration. A similar cytokine profile was detected in our murine model of TNF-independent UC (Winnie–TNF–knockout [KO]).24 Thus, we evaluated whether anakinra administration to Winnie-TNF-KO mice could reduce intestinal signs of chronic inflammation. Our results highlight the beneficial effects of anakinra administration, characterized by a significant decrease in interferon (IFN)γ production by mucosal CD8+ T cells. Mice affected by TNF-independent UC resemble a subpopulation of UC patients that currently are defined as primary nonresponders after months of ineffective IFX administration; they represent a novel model to study this important patient population. The results of the present study pave the way to clinical trials based on anakinra administration to a subgroup of primary nonresponder UC patients, characterized by high IL1β in sera and colonic biopsy specimens.

Results

Increased IL1β in IBD Patients Defined as Primary Nonresponders

A cohort of 30 UC patients was enrolled and started IFX therapy. Patients’ sera were collected and stored before the...
first infusion (T0) and 12 weeks after the first infusion (T1). At T1, patients’ response to IFX was assessed by routine endoscopic examination. All patients had similar serum levels of C-reactive protein; 18 patients were classified as responders to the biologic, while 12 were nonresponders.

Patients’ sera were analyzed by multiplex for 13 target cytokines. As shown in Figure 1A, the nonresponder group was characterized by a significantly higher concentration of circulating IL1β compared with responders, even at the T0 time point, suggesting a role for IL1β as a predictor for IFX primary nonresponders. Specifically, the IL1β mean concentration in sera was 14.23 ± 4.75 pg/mL in nonresponder patients compared with 2.66 ± 1.73 pg/mL in the responder group at T0, and 14.53 ± 5.51 pg/mL in nonresponders vs 1.98 ± 0.87 pg/mL in responders were measured at T1 (Figure 1A). Results obtained from the analysis of 12 other cytokines, including TNF and IL6, did not support any predictive role regarding primary nonresponder patients. Serum levels of IL2 were below the standard limit of detection in all of the analyzed samples.

To better understand the differences observed between responder and nonresponder patients, and to determine if a correlation exists among all cytokines, we performed a linear regression analysis between circulating levels of IL1β vs all other cytokines analyzed in both the responder and nonresponder groups. No correlation was detected among all circulating cytokines in the responder group at T0 and T1 (Tables 1 and 2, respectively), as well as in the nonresponders at T0 (Table 3), while a positive correlation was detected for 9 analyzed cytokines (TNF, IFNγ, IL9, IL12p70, IL10, IL17A, IL4, IL5, and granulocyte-macrophage colony-stimulating factor [GM-CSF]) at T1 only in the nonresponder group (Table 4).

In further support of the predictive role of IL1β in nonresponder patients, we obtained intestinal biopsy specimens from the same cohort used for cytokine assessment (Figure 1B). Immunohistochemistry (IHC) staining showed that IL1β and caspase 8-positive cells were detectable in 75% and 83% of nonresponder patients, respectively, while a dramatic reduction of IL1β-positive (46.2%), but not caspase 8-positive (91.7%), cells were observed in biopsy specimens from responder patients. A significant correlation was observed between the inflammatory score and the percentage of caspase 8-positive cells in nonresponder as well as in the responder patients (Figure 2).

Increased IL1β Production by DCs in the Absence of TNF

To investigate a possible correlation between IL1β and TNF secretion in the inflammatory response, we isolated and cultured bone marrow–derived dendritic cells (BMDCs) from wild-type (WT) and TNF-KO mice. In the TNF-KO BMDC media, we detected significantly higher concentrations of IL1β, IL16, IL9, C-C motif chemokine ligand (CCL)-2, CCL-3, CCL-4, CCL-5, CCL-11, and granulocyte colony-stimulating factor (Figure 3A), while we observed TNF, IFNγ, IL6, IL10, IL12, and keratinocyte-derived chemokine to be reduced significantly (Figure 3B). In both groups, lipopolysaccharide (LPS) administration did not result in increased secretion of IL4, IL13, and GM-CSF within each genotype, but TNF-KO BMDCs produced higher quantities of IL4 and GM-CSF (significantly) than WT BMDCs, even in the absence of LPS (Figure 3C).

Anakinra Administration Successfully Suppresses Colonic IL1β Production

We hypothesize that a percentage of primary nonresponder UC patients are characterized by a TNF-independent disease and abundant IL1β secretion. To better explore this hypothesis, we took advantage of our previously generated murine model of TNF-independent UC, obtained by crossing the progressive and spontaneous UC model Winnie25,26 with the commercial strain TNF-KO (Winnie-TNF-KO).24 Immediately after weaning, we observed significantly increased expression of IL1β by measuring the messenger RNA extracted from colonic tissues of Winnie-TNF-KO compared with controls (WT mice and parental strains).24

All of these experimental observations indicated that, in the Winnie model, the absence of TNF does not suppress colonic inflammation, and vice versa this is fostered through the production of the inflammatory mediator IL1β. Therefore, our hypothesis is that blocking IL1 could be an effective therapeutic option for TNF-independent UC patients.

To evaluate whether blocking IL1 could protect Winnie-TNF-KO mice from UC-like colitis, we used an experimental protocol of anakinra administration, a recombinant IL1-receptor antagonist. Winnie and WT mice were used as control. As shown in Figure 4A, we treated adult mice that were 16 weeks of age (N = 9 for each group) with daily intraperitoneal injections of 60 mg/kg anakinra or vehicle (phosphate-buffered saline [PBS]) for 2 weeks. Before the treatment and every 2 days, we recorded mice body weight and the disease activity index (DAI) score. As expected, no toxic side effects were observed with anakinra administration; indeed, mice gained weight regularly during the 2 weeks of treatment (Figure 4B), with a similar trend to vehicle administration. The absence of any adverse side effects related to anakinra administration was shown previously.27 Moreover, in our previous studies, Winnie mice showed a reduced weight at weaning compared with their WT counterparts, and this difference persisted also at adult age; nevertheless, Winnie mice were able to gain weight during life.24,26 The DAI score recorded was almost 0 in the WT mice at each time point. Instead, in Winnie mice treated with vehicle, the initial DAI score was 1.3 ± 0; at the end of treatment, it decreased to 1.0 ± 0.3. Similarly, Winnie mice in the anakinra group had an initial DAI score of 1.6 ± 0.3 that decreased to 1.0 ± 0.3. Lastly, for vehicle-treated Winnie-TNF-KO mice, the DAI score was 2.3 ± 0.3 at day 0; at the end of treatment, it decreased to 1.6 ± 0.3, while the anakinra group started from a DAI score of 1.6 ± 0.3, which decreased to 0.3 ± 0.3 at the end of treatment (Figure 4C).

At the end of treatment, mice were killed, and colons were harvested for subsequent histologic analysis. As shown in Figure 4D, the colon length in WT mice was comparable after treatment with anakinra or vehicle, as well as stool
A

|   | TNF | IFNγ | IL-6 | IL-1β |
|---|-----|------|------|-------|
|   | pg/mL | pg/mL | pg/mL | pg/mL |
|   | 0 | 200 | 1000 | 110 |
|   | 400 | 600 | 50 | 30 |

|   | IL-9 | IL-10 | IL-12 | IL-17A |
|---|------|-------|-------|--------|
|   | pg/mL | pg/mL | pg/mL | pg/mL |
|   | 0 | 200 | 1000 | 80 |
|   | 400 | 600 | 40 | 20 |

|   | IL-4 | IL-5 | IFNα | GM-CSF |
|---|------|------|------|--------|
|   | pg/mL | pg/mL | pg/mL | pg/mL |
|   | 0 | 200 | 1000 | 25 |
|   | 400 | 600 | 40 | 5 |

B

**IL-1β**

**Caspase 8**

**Responder**

**Non-responder**

![Images of tissue sections for responder and non-responder](image)
consistency. Both Winnie and Winnie-TNF-KO mice treated with vehicle showed colonic fibrosis and watery stool. After anakinra injections, the colon length increased slightly. The treatment with anakinra also ameliorated the ratio between colon weight and total body weight (Figure 4E), even if the differences observed did not reach statistical significance. Instead, no differences were observed in the ratio between colon length and total body weight (Figure 4F).

Anakinra efficiency in Winnie-TNF-KO mice was confirmed by quantitative determination of neutrophil gelatinase-associated lipocalin (N-GAL) in feces. As shown in Figure 4G, the fecal N-GAL level was almost 0 for WT mice treated with vehicle or anakinra. On the contrary, in anakinra-treated Winnie mice, the fecal N-GAL was 26.2 ng/g feces, which was significantly lower than vehicle-treated Winnie mice (18.60 ± 37.4 ng/g feces). Similarly, in Winnie-TNF-KO mice the level of fecal N-GAL was significantly lower in the anakinra group compared with vehicle (72.2 ± 20.2 vs 149.2 ± 19.1 ng/g feces, respectively).

IHC of the distal colon showed positive IL1β staining in Winnie-TNF-KO mice, while it was absent in all control strains (WT and Winnie mice) (Figure 4H). IL1β decreased significantly after anakinra administration in Winnie-TNF-KO mice. However, the signal for IL1β still was present and well defined as ubiquitous positivity of the intestinal endoluminal border (Figure 4H, enlargement). In vehicle-treated Winnie-TNF-KO, IL1β IHC showed a diffuse positivity in crypts, lymphoplasma cellular inflammatory cells, and surface desquamating cells (Figure 4H). Likewise, the expression of caspase 8 was decreased significantly in the intestinal tissues of anakinra-treated Winnie-TNF-KO mice compared with controls (Figure 4I). Positive cells were located only on the endoluminal secretory side. In vehicle-treated Winnie-TNF-KO, caspase 8 immunolocalization showed strong positivity in the cytoplasm and/or nucleus of colonic epithelial cells, with the highest expression in the crypts and at the tip of erosion areas (Figure 4I).

Approximately 0.5 cm of tissue from the terminal colon was cultured in vitro, the supernatant was collected 24 hours later, and analyzed by enzyme-linked immunosorbent assay (ELISA). Supernatants from the colon culture of Winnie-TNF-KO mice were enriched in IL1β and IFNγ if compared with the parental strain Winnie (Figure 5). When the colon was obtained from anakinra-treated mice, a decrease in both cytokines could be observed, significant only for IL1β. Similarly, IL6 and IL18 appear differently secreted in our experimental groups, even though there was no significance (Figure 5).

**Anakinra Reduces Gene Expression and Secretion of Inflammatory Cytokines**

Gene expression analysis by Quantitative Polymerase Chain Reaction of the distal colon of treated mice confirmed significant IL1α and IL1β reduction in Winnie-TNF-KO after anakinra administration. Moreover, inflammatory UC markers S100a8 and S100a9 and C3 expression levels decreased after anakinra administration in Winnie-TNF-KO mice (Figures 6 and 7). Inflammatory genes related to the IL1 pathway, IL18 and IL33, were not affected by anakinra administration, while Ifnγ expression was reduced significantly in Winnie-TNF-KO. Similarly, the Socs3/Stat3/Il6 pathway expression was reduced in Winnie-TNF-KO after anakinra administration. It is important to notice that the expression of numerous genes, including Il1α, Il1β, Socs3, Stat3, and Il6, was elicited in Winnie mice after anakinra administration (Figures 6 and 7).

We then performed fluorescence-activated cell sorting analysis of mesenteric lymph node (MLN) and lamina propria (LP) cells to evaluate the effects of anakinra administration on lymphocyte polarization. In particular, we analyzed the production of TNF, IFNγ, IL9, and IL17A by CD4⁺ and CD8⁺ cells. Figure 8 shows the experimental scheme used. In MLN of Winnie-TNF-KO mice treated with anakinra, there was an increased percentage of CD4⁺ cells (39.6% ± 1.5%) compared with the WT group treated with anakinra (31.4% ± 1.4%), while the percentages of CD8⁺ cells were similar in all experimental conditions (Figure 9A). Treatment with anakinra significantly reduced secretion of IFNγ from both CD4⁺ T cells in all of the experimental groups and, to a greater extent, from CD8⁺ T cells in Winnie-TNF-KO mice (Figure 9). Interestingly, in Winnie-TNF-KO mice, the percentage of CD8⁺ IFNγ⁺ cells decreased from 5.0% ± 1.1% with the vehicle to 2.3% ± 0.4% with anakinra. Moreover, in Winnie-TNF-KO mice, anakinra significantly reduced the percentage of CD4⁺ IL9⁺ T cells (from 1.7% ± 0.3% with the vehicle to 0.7% ± 0.1% with anakinra) (Figure 9B). Lastly, in anakinra-treated Winnie mice, the percentage of CD4⁺ IL17A⁺ cells decreased, while no relevant differences were observed for TNF in both CD4⁺ and CD8⁺ cells from WT and Winnie mice (Figure 9B). Lastly, we detected significantly increased percentages of CD4⁺ CD25⁺ forkhead box P3 (Foxp3⁺) regulatory T cells only in WT mice MLNs after anakinra administration (12.1% ± 0.5% with the vehicle vs 13.5% ± 0.4% with anakinra) (Figure 10).

In the LP, the absence of TNF resulted in a sharp increase in the percentage of IFNγ⁺ CD8⁺ cells, from 45.6% ± 1.6% in Winnie to 63.7% ± 1.2% in Winnie-TNF-KO mice (Figure 11). Anakinra administration was able to reduce IFNγ⁺ CD8⁺ cells in both models. In Winnie mice, the percentage decreased to 37.2% ± 2.5%, while in Winnie-TNF-KO mice it decreased to 44.7% ± 4.5%. In LP of Winnie mice, the percentage of CD4⁺ IFNγ⁺ cells was similar in both the vehicle and anakinra groups (7.8% ± 0.3% vs 8.0% ± 1.2%, respectively). In Winnie-TNF-KO mice, instead, it decreased significantly from 15.2% ± 1.2% in the vehicle group to 5.7% ± 1.4% in the anakinra-treated mice (Figure 11B).
### Table 1. Correlation Between the Concentrations of Circulating IL1β and All Other Cytokines in the Responder Group at T0

| Parameter | IFNα | IL9 | TNF | IFNγ | IL6 | IL12p70 | IL10 | IL17A | IL4 | IL5 | GM-CSF |
|-----------|------|-----|-----|------|-----|---------|------|-------|-----|-----|--------|
| Number of XY pairs | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 |
| Pearson r | -0.04885 | -0.09036 | -0.1001 | -0.09567 | -0.1099 | -0.1210 | -0.06357 | -0.2456 | -0.1330 | -0.1207 | -0.1133 |
| 95% CI | -0.5043 to 0.4279 | -0.5348 to 0.3932 | -0.5417 to 0.3849 | -0.536 to 0.3887 | -0.5487 to 0.3764 | -0.5565 to 0.3667 | -0.5152 to 0.4157 | -0.6393 to 0.2500 | -0.5649 to 0.3560 | -0.5563 to 0.3670 | -0.5511 to 0.3734 |
| P value (2-tailed) | .8474 | .7214 | .6927 | .7057 | .6642 | .6325 | .8021 | .3259 | .5987 | .6334 | .6544 |
| P value summary | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS |
| Is the correlation significant? (α = .05) | No | No | No | No | No | No | No | No | No | No | No |
| R² | 0.002386 | 0.008166 | 0.01002 | 0.009153 | 0.01208 | 0.01453 | 0.004041 | 0.06033 | 0.01770 | 0.01456 | 0.01284 |

**NOTE.** All data were analyzed using the Pearson correlation test.

### Table 2. Correlation Between the Concentrations of Circulating IL1β and All Other Cytokines in the Responder Group at T1

| Parameter | IFNα | IL9 | TNF | IFNγ | IL6 | IL12p70 | IL10 | IL17A | IL4 | IL5 | GM-CSF |
|-----------|------|-----|-----|------|-----|---------|------|-------|-----|-----|--------|
| Number of XY pairs | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 |
| Pearson r | -0.04982 | -0.1702 | -0.1730 | -0.1228 | -0.1628 | -0.1927 | -0.02136 | -0.3663 | -0.1353 | -0.2177 | -0.2260 |
| 95% CI | -0.5050 to 0.4271 | -0.5902 to 0.3224 | -0.5922 to 0.3198 | -0.5578 to 0.3651 | -0.5853 to 0.3292 | -0.6052 to 0.3014 | -0.4853 to 0.4501 | -0.7116 to 0.1214 | -0.5665 to 0.3540 | -0.5615 to 0.2775 | -0.6268 to 0.2694 |
| P value (2-tailed) | .8444 | .4996 | .4923 | .6275 | .5187 | .4435 | .9329 | .1349 | .5925 | .3855 | .3672 |
| P value summary | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS |
| Is the correlation significant? (α = .05) | No | No | No | No | No | No | No | No | No | No | No |
| R² | 0.002482 | 0.02896 | 0.02994 | 0.01507 | 0.02650 | 0.03715 | 0.0004563 | 0.1342 | 0.01831 | 0.04739 | 0.05107 |

**NOTE.** All data were analyzed using the Pearson correlation test.
### Table 3. Correlation Between the Concentrations of Circulating IL1β and All Other Cytokines in the Nonresponder Group at T0

| Parameter | IFNα  | IL9  | TNF  | IFNγ  | IL6  | IL12p70 | IL10 | IL17A | IL4  | IL5  | GM-CSF |
|-----------|-------|------|------|-------|------|---------|------|-------|------|------|--------|
| Number of XY pairs | 12    | 12   | 12   | 12    | 12   | 12      | 12   | 12    | 12   | 12   | 12     |
| Pearson r | -0.2126 | 0.1876 | -0.02277 | 0.3847 | -0.1919 | 0.2611 | 0.2071 | 0.2167 | 0.2219 | 0.3949 | 0.3015 |
| 95% CI    | -0.7011 to 0.4116 | -0.4330 to 0.6876 | -0.5891 to 0.5585 | -0.2430 to 0.7853 | -0.6899 to 0.4294 | -0.3681 to 0.7282 | -0.4165 to 0.6981 | -0.4080 to 0.7033 | -0.4035 to 0.7060 | -0.2316 to 0.7899 | -0.3295 to 0.7464 |
| P value (2-tailed) | .5071 | .5592 | .9440 | .2169 | .5501 | .4125 | .5185 | .4987 | .4883 | .2039 | .3408 |
| P value summary | NS    | NS   | NS   | NS    | NS   | NS      | NS   | NS    | NS   | NS   | NS     |
| Is the correlation significant? (α = .05) | No    | No   | No   | No    | No   | No      | No   | No    | No   | No   | No     |
| R²        | 0.04520 | 0.03521 | 0.0005185 | 0.1480 | 0.03684 | 0.06815 | 0.04287 | 0.04697 | 0.04922 | 0.1559 | 0.09093 |

NOTE. All data were analyzed using the Pearson correlation test.

### Table 4. Correlation Between the Concentrations of Circulating IL1β and All Other Cytokines in the Nonresponder Group at T1

| Parameter | IFNα  | IL9  | TNF  | IFNγ  | IL6  | IL12p70 | IL10 | IL17A | IL4  | IL5  | GM-CSF |
|-----------|-------|------|------|-------|------|---------|------|-------|------|------|--------|
| Number of XY pairs | 12    | 12   | 12   | 12    | 12   | 12      | 12   | 12    | 12   | 12   | 12     |
| Pearson r | -0.1790 | 0.5854 | 0.7472 | 0.7156 | 0.5305 | 0.7483 | 0.7225 | 0.6496 | 0.6983 | 0.7386 | 0.6570 |
| 95% CI    | -0.6828 to 0.4403 | 0.01722 to 0.8678 | 0.3033 to 0.9246 | 0.2403 to 0.9141 | -0.06261 to 0.8467 | 0.3056 to 0.9250 | 0.2537 to 0.9164 | 0.1206 to 0.8913 | 0.2075 to 0.9083 | 0.2857 to 0.9218 | 0.1333 to 0.8939 |
| P value (2-tailed) | .5778 | .0455 | .0052 | .0089 | .760 | .0051 | .0080 | .0222 | .0115 | .0061 | .0203 |
| P value summary | NS    | *    | **   | **    | NS   | *      | **   | *     | *    | **   | **     |
| Is the correlation significant? (α = .05) | No    | Yes  | Yes  | Yes   | No   | Yes    | Yes  | Yes   | Yes  | Yes  | Yes    |
| R²        | 0.03203 | 0.3427 | 0.5583 | 0.5121 | 0.2814 | 0.5600 | 0.5220 | 0.4220 | 0.4877 | 0.5455 | 0.4317 |

NOTE. All data were analyzed using the Pearson correlation test. *P < .05, **P < .01
Discussion
Despite the introduction of numerous drugs and biological treatments for UC, primary nonresponders to anti-TNF represent a major concern for gastroenterologists concerning an appropriate approach to effectively treat this patient population. The absence of reliable predictors for driving the most effective therapeutic choice leaves no other options than conventional drug administration and frequent follow-up evaluation with serologic and endoscopic evaluation. Besides this, the increasing presence in the IBD

Figure 2. Linear regression between inflammatory score and the percentage of IL1β or caspase 8 (CASP8)-positive cells in the (A) nonresponder (N = 8) and (B) responder (N = 12) groups at T0.

Figure 3. Murine BMDC cytokine (CK) and chemokine (CC) profiles after LPS exposure determined by multiplex assay. BMDCs from WT and TNF-KO mice were exposed to LPS for 24 hours (N = 8 for each group). Dark grey and blue circles represent the CK or CC level from WT and TNF-KO BMDCs, respectively; light grey and light blue circles show the CK and CC levels after LPS exposure. (A) CK and CC were increased in the TNF-KO BMDCs. (B) CK and CC were decreased in the TNF-KO BMDCs. (C) There was no effect in CK and CC secretion mediated by LPS stimulation. *P < .05, **P < .01, and ***P < .001. "WT versus WT + LPS; "TNF-KO versus TNF-KO + LPS; "WT + LPS versus TNF-KO + LPS. G-CSF, granulocyte colony-stimulating factor; KC, keratinocyte-derived chemokine.
pipeline of innovative biological therapies targeting different cytokines and immune processes raises a clear need for predictors of efficacy for anti-TNF treatment.

Indeed, the right drug and specific treatment strategy for each patient is the end goal for personalized medicine.6

In the present study, we analyzed the circulating cytokine profiles of a cohort of 30 UC patients including 18 responders and 12 primary nonresponders. Significant differences in IL1β were associated with primary nonresponse to IFX administration, confirming the previous report describing IL1β as a possible predictive factor to identify a subpopulation of primary nonresponders.12,22,23 IL1β and caspase 8 were detectable by IHC in biopsy specimens from a percentage of nonresponder patients. Importantly, IL1β-positive cells were absent in biopsy specimens from 25% of nonresponder patients, independently from the inflammatory score. These results indicate that IHC staining for IL1β-positive cell assessment may be an important predictive test for deciding the most appropriate biological drug administration.

In this context, in our recent study, we created an animal model of TNF-independent intestinal inflammation (Winnie-TNF-KO) in which we observed a significant increase in colonic IL1β.24 Notably, this model introduced the concept of TNF-independent colitis, which may represent a significant number of primary nonresponder patients. Nonetheless, this does not infer that primary nonresponders are TNF negative, but underlines the possibility that TNF may not be the most appropriate target in a subpopulation of UC patients.

IL1β can be efficiently blocked by anakinra, a recombinant form of IL1Ra, successfully used for the treatment of rheumatoid arthritis.17 Numerous studies have reported the importance of IL1β and IL1Ra genetic polymorphism in IBD,29,29 but only recently was a clinical trial based on anakinra administration to patients affected by a severe form of acute severe ulcerative colitis approved.30 Administration of anakinra to UC patients may result in nonsignificant disease remission if the patient cohort is not preliminarily selected. Our results indicate that only a percentage of primary nonresponder UC patients is characterized by increased IL1β in sera and colonic biopsy specimens. Thus, these parameters should be used as inclusion criteria for patient enrollment in a new clinical trial based on anakinra administration to primary nonresponder UC patients.

The Winnie model is a mild but progressive model of UC-like colitis, showing several features of the human disease. Although the histologic features of UC-like colitis are modest, the presence of inflammation can be detected by gene expression, cytokine profile, and dysbiosis.25,26 The Winnie inflammatory pathway is not triggered by TNF. Indeed, the intestinal tract of Winnie-TNF-KO mice is affected by pathologic features similarly to Winnie.24 We noted potential negative feedback between TNF and IL1β, suggesting an inverse correlation between TNF and IL1β secretion.

With the intent of providing a rationale for a new therapeutic option suited for a subgroup of anti-TNF primary nonresponder UC patients, characterized by increased circulating levels of IL1β, we evaluated the effects of anakinra administration to Winnie-TNF-KO mice. Anakinra injections significantly reduced the DAI score in Winnie-TNF-KO mice compared with their vehicle-treated counterparts and Winnie mice, this provided useful insights into the positive effects that IL1β blockade has on colitis. Moreover, immunohistology examination showed that IL1β could be detected only in the colonic tissue of Winnie-TNF-KO mice, reinforcing the idea of TNF-mediated negative feedback for IL1β secretion. Intraperitoneal administration of anakinra efficiently blocked intestinal IL1β and caspase 8 in Winnie-TNF-KO.

Caspase 8 activity and function are regulated through shutting between the nucleus and the cytoplasm.31–33 The nuclear active form of caspase 8 mediates the transcription of a broad array of genes involved in cell-cycle inhibition and apoptosis.34–36 Recently, it was shown that caspase 8 is crucially involved in the induction of pro-IL1β synthesis and processing via both noncanonical and canonical pathways.37

In the present study, in anakinra-treated Winnie-TNF-KO mice, caspase 8 expression was detected only in the cytoplasm, while in vehicle-treated Winnie-TNF-KO mice, caspase 8 also was present in the nucleus. In line with these results, IL1β was detected ubiquitously in vehicle-treated Winnie-TNF-KO mice, while only present at the desquamating tip of anakinra-treated Winnie-TNF-KO mice, suggesting initiation of homeostatic reepithelization.

The intracellular staining of MLN CD4+ and CD8+ T cells shows that Winnie-TNF-KO mice are characterized by a higher frequency of IFNγ+ and IL9+ CD4+ T cells when compared with Winnie, suggesting a relevant role for T helper 9 (Th9) cells in this TNF-independent model of UC.38 Anakinra administration was able to reduce the percentage of IFNγ+ CD4+ T cells in Winnie, while the percentage of TNF+ CD4+ T cells was unchanged. These data suggest that, at least under these experimental conditions, TNF can influence IL1β production, but the opposite is not true, or that in TNF competent models, IL1β is not expressed sufficiently in the mucosal tissues.

When compared with the parental strain Winnie, the percentage of IFNγ+ CD8+ T cells was higher in Winnie-TNF-KO mice, suggesting a pivotal role for cytotoxic T cells in the TNF-independent intestinal inflammation. The percentage of IFNγ+ CD8+ T cells sharply decreases after anakinra administration, revealing the importance of IL1β in sustaining this crucial inflammatory pathway. As reported by Corridoni et al,39 there is extensive heterogeneity of CD8+ T cells playing a primary role in UC. These results are even more dramatic in the lamina propria, where approximately 64% of CD8 cells are IFNγ+ in Winnie-TNF-KO mice. Anakinra administration was able to reduce IFNγ+ CD8+ cells to 45%, the same percentage of IFNγ+ CD8+ observed in Winnie mice.

Although we did not characterize the presence of IL26+ CD8+ T cells in the intestinal mucosa, the axis between IL1β and IL26 already is known and potentially may contribute and explain the effects of anakinra administration in TNF-independent UC.40
ELISA assays of the colonic tissue culture showed that, in the absence of TNF, the inflammatory pathway can switch to an IL1β- and IFNγ-dependent inflammation. Anakinra administration was able to reduce IL1β release both in Winnie and Winnie-TNF-KO. Finally, analysis of the distal colon showed a reduction of several inflammatory markers, including Il1α, Il1β, Ifnγ, and C3, in Winnie-TNF-KO mice. The results also indicated increased expression of these genes in Winnie mice after anakinra administration. In the absence of TNF, the IL1β inflammatory pathway becomes prevalent, thus the IL1 sequestration strategy efficiently blocks the inflammatory cascade. Surprisingly, the expression of some inflammatory mediators is elicited by anakinra in Winnie mice. This is particularly evident for the Socs3/Stat3/Il6 expression pathway that is induced by IL1 sequestration in Winnie. This may be owing to the need to compensate for the blockade of activity of both IL1α and IL1β by the IL1-receptor antagonist. Overall, the gene expression pathway obtained from the distal colon indicates a reduced intestinal inflammation in anakinra-treated mice. Our results highlight the beneficial effects of anakinra administration, particularly in mice affected by TNF-independent UC, a feature that may characterize a subpopulation of UC patients. Precision medicine relies on efficient patient stratification. Adjustments to the therapeutic strategies may be driven by the objective assessment of circulating inflammatory markers, even if in our mice models this analysis was inconsistent owing to the numerous samples below detection levels. Another important approach may be represented by the noninvasive analysis of patients’ fecal material, which could be considered a reliable option to adjust patients’ therapies. In our model, N-GAL assessment was able to indicate the mouse response to anakinra. UC patients share similar effects triggered by different genetic and environmental factors. TNF is clearly among the most important factor involved in the inflammatory cascade causing uncontrolled chronic inflammation. Nonetheless, TNF-independent UC is an event likely unnoticed, even for primary nonresponders, to anti-TNF therapy. Our results indicate that circulating levels of IL1β can be used as a

Figure 5. Cytokine profile of colonic tissue culture from Winnie and Winnie-TNF-KO mice treated with vehicle or anakinra (N = 3 for each group). **P < .005, ***P < .001.

Figure 4. (See previous page). Macroscopic features and measurements of colonic parameters at the end of treatment with anakinra. (A) Schematic representation of the experimental design. WT, Winnie and Winnie-TNF-KO mice were treated with IP injection of anakinra or vehicle for 14 days (N = 9 for each group). (B) Effect of anakinra or vehicle on mice body weight from day 0 until the end of treatment. (C) DAI score recorded for Winnie and Winnie-TNF-KO mice treated with anakinra or vehicle. (D) Representative images of whole colons for each experimental group. (E and F) Measurement of colon weight/body weight and colon length/body weight indices (%), respectively. (G) Detection of N-GAL level in feces of WT, Winnie, and Winnie-TNF-KO mice treated with anakinra or vehicle. (H and I) IHC analysis for IL1β and caspase 8, respectively, in formalin-fixed, paraffin-embedded tissues obtained from WT, Winnie, and Winnie-TNF-KO mice treated with anakinra or vehicle. (10× and magnified 20× on the right). *P < .05, **P < .01, and ***P < .001.
predictor for primary nonresponder patients, who may be enrolled in clinical trials. Our data also highlight the need for a thorough patient selection in future clinical trials based on IL1 sequestration to primary nonresponder UC patients and routine analysis of fecal material inflammatory markers to allow real-time adjustments to the therapeutic strategy.

The use of anakinra in inflammatory disorders related to TNF dysfunction already has produced promising responses, including for the treatment of TNF Receptor-Associated Periodic Syndrome, an autoinflammatory disorder caused by a mutant TNF receptor that is not transported to the cell surface efficiently.42,43 TNF Receptor-Associated Periodic Syndrome is characterized by increased inflammatory cytokine secretion, including IL1, underscoring the potential negative feedback mechanism(s) between TNF and IL1.44,45 Although the axis between TNF and IL1β may require further investigation, our results lay the foundation for a new concept of TNF-independent UC that requires innovative approaches to suppress chronic inflammation favoring UC remission.

Materials and Methods

Ethical Considerations

The study protocols on human subjects were conducted in accordance with the principles of the Declaration of Helsinki and approved by the local ethics committee (ID: 2383 Comitato Etico Fondazione Policlinico Universitario
Figure 7. Gene expression analysis of the 88 genes expressed in the distal colon of WT (N = 3), Winnie (N = 3), and Winnie-TNF-KO (N = 4) mice treated with vehicle or anakinra. *p < 0.05 (Anakinra vs vehicle)
“A. Gemelli” Istituto di Ricovero e Cura a Carattere Scientifico and number 333 National Institute of Gastroenterology “S. de Bellis”.

Our investigations were performed under the relevant animal protocol, which was approved by the Institutional Animal Care Committee of the National Institute of Gastroenterology “S. de Bellis” (Organismo Preposto al Benessere degli Animali [OPBA]). All of the animal experiments were performed according to the national guidelines of Italian Directive 26/2014 and approved by the Italian Animal Ethics Committee of Ministry of Health–General Directorate of Animal Health and Veterinary Drugs (Direzione generale della sanità’ animale e dei farmaci veterinari [DGSAF] - Protocollo 768/2015-PR 27/07/2015). All animals were maintained in a controlled environment (20°C–22°C, 12-hour light and 12-hour dark cycle, and 45%–55% relative humidity).

**Murine Models**

WT mice were purchased from Jackson Laboratories, (Bar Harbor, ME; C57BL/6, stock no.: 000664).

The murine transgenic line Winnie-TNF-KO was created in our laboratory previously by breeding heterozygous mice from the TNF knockout line and heterozygous Winnie mice.

The TNF knockout mice were purchased from Jackson Laboratories (B6.129S-Tnftm1Gkl/J, stock no: 005540), while Winnie mice were obtained from the University of Tasmania, Launceston, TAS, Australia.

Body weight, stool consistency, and rectal bleeding were recorded daily. Mice were killed 14 days after the first intraperitoneal (IP) injection, and the colon was explanted to evaluate the clinical severity of colitis. Colon length and weight were measured as indicators of colonic inflammation. The colon/body weight indices were calculated as the ratio of the colon wet weight and the total body weight and as the ratio of the colon length and the total body weight of each mouse. The DAI was determined by scoring changes in body weight (0–4), stool consistency (0–4), and occult blood (0–4).

**Treatment With IL1-Receptor Antagonist**

Adult mice age 16 weeks were treated with anakinra (Kineret; Swedish Orphan Biovitrum AB, Stockholm, Sweden, 60 mg/kg in 0.1 mL PBS) or vehicle (0.1 mL PBS) via IP injections every day for 2 weeks. These doses of anakinra were chosen because they are within the range of doses that have been shown previously to be efficacious in other models of disease in mice.46,47 No signs of any adverse side effects were recorded with these doses of anakinra.

**Histologic Examination**

Tissue sections from the proximal, medial, and distal colon were fixed in 10% buffered formalin and embedded in paraffin. Sections (3 μm) were deparaffinized in xylene, rehydrated with ethanol series and water, and washed in PBS. H&E staining was performed on the sections using standard techniques. Images were acquired using a Nikon Eclipse Ti2 microscope (Nikon, Tokyo, Japan).

**IHC**

IHC analyses for IL1β and caspase 8 were performed in the formalin-fixed paraffin-embedded tissues obtained from WT, Winnie, and Winnie-TNF-KO mice treated with anakinra or vehicle, and biopsy specimens from UC patients at the beginning of anti-TNF treatment. Distal colon sections (4 μm) were freshly cut and dried at 60°C for 30 minutes. IHC analysis was performed in sections after deparaffinization for 30 minutes and then rehydration in grades of alcohol. Antigen retrieval was performed at 90°C for 20 minutes with sodium citrate buffer (Sigma-Aldrich, St. Louis, MO). To assess the IL1β and caspase 8 staining used for the present study, the samples were blocked with blocking buffer, incubated with IL1β (Cleaved Asp116, polyclonal, Thermo
Figure 9. (A) Representative density plot analysis of intracellular staining of IFNγ from CD8⁺ T cells in MLN of WT, Winnie, and Winnie-TNF-KO mice, treated with vehicle or anakinra. (B) Frequencies of CD4⁺ and CD8⁺ T cells and intracellular staining of TNF, IFNγ, IL9, and IL17A from CD4⁺ and CD8⁺ T cells, in the MLNs of WT, Winnie, and Winnie-TNF-KO mice treated with anakinra or vehicle. *P < .05, **P < .01, and ***P < .001.
Fisher Scientific, Waltham, MA) and caspase 8, polyclonal antibody (Thermo Fisher Scientific, Waltham, MA) using a 1:100 dilution (2 h, 22°C), followed by horseradish-peroxidase–conjugated goat anti-rabbit IgG (Heavy and Light chains) secondary antibody at a dilution of 1:500 for 30 minutes at room temperature (Thermo Fisher Scientific). Chromogenic detection was performed using the Metal Enhanced DAB Substrate Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Negative control sections were incubated without the primary antibody in citrate buffer. All sections were counterstained with hematoxylin. Images were taken on a Nikon Eclipse Ti2 microscope (Nikon) at a magnification of 20×. The histologic score was based on the percentage of inflammatory cells present in the mucosa: a score of 0 indicated no inflammatory cells; 1 indicated 1%–25% of cells; 2 indicated 25%–50% of cells; 3 indicated 50%–75% of cells; and 4 indicated 75%–100% of inflammatory cells.

Cytofluorimetric Assay

To obtain LP cells, murine colons were cut into small segments (1-cm long), washed with Dulbecco’s Phosphate-Buffered Saline (DPBS) 1× ( Gibco, Waltham, MA) and 2.5 mmol/L EDTA (Ambion, Thermo Fisher Scientific) to separate from epithelial cells, and digested with collagenase type IV and DNase I ( Sigma Aldrich) for 30 minutes at 37°C after GentleMACS processing. Resulting cell suspensions were pelleted by centrifugation, washed with DPBS 1× + 0.5 mmol/L EDTA, and passed through 100-μm and 30-μm cell strainers (Miltenyi Biotec, Bergisch Gladbach, Germany) and washed with DPBS (Gibco) + 0.5% bovine serum albumin (BSA; Sigma-Aldrich).

MLNs were isolated from mice treated with anakinra or vehicle. MLNs were passed through a 30-μm cell strainer (Miltenyi Biotec) to obtain a single-cell suspension and washed with DPBS (Gibco) + 0.5% BSA (Sigma-Aldrich).

**Foxp3 staining.** Single-cell suspensions were stained with CD4–FITC (fluorescein isothiocyanate) and CD25–phycoerythrin (PE) (Miltenyi Biotec). Cells were permeabilized with the Foxp3 Fixation/Permeabilization Kit (eBioscience, San Diego, CA) and washed with PERM Buffer (eBioscience). Finally, cells were stained with Foxp3-Allophycocyanin (APC) (Miltenyi Biotec), according to the manufacturer’s instructions.

**T-cell intracellular staining.** T cells from LP and MLN of mice treated with anakinra or vehicle were cultured with a 500× Cell Stimulation Cocktail (eBioscience) for 12 hours, washed with DPBS + 0.5% BSA, and stained with CD4–FITC and CDB-APC-Vio700 (Miltenyi Biotec). After washing, cells were permeabilized with BD CytoFix/CytoPerm Fixation/Permeabilization Kit (BD Biosciences, Franklin Lakes, NJ), washed with PERM Buffer, and stained with IL9–PE, IL17A–APC, TNF–PE, and IFNγ–APC according to the manufacturer’s instructions (Miltenyi Biotec).

For both stainings, Flow Cytometer acquisition was performed using NAVIOS (Beckman Coulter, Brea, CA). Flow cytometer analysis was performed using Kaluza Software 1.5 (Beckman Coulter).

**RNA Extraction and Quantitative Polymerase Chain Reaction Analysis**

Total RNA was isolated from the distal colon of mice treated with anakinra or vehicle. The RNA was extracted using TRIzol (Thermo Fisher Scientific) according to the manufacturer’s instructions. Total RNA (1 μg) was reverse-transcribed using an iScript complementary DNA Synthesis kit (Bio-Rad, Hercules, CA) with random primers for complementary DNA synthesis. Gene expression of 88 genes was performed using the Colitis, Ulcerative Tier 1 M96 (Bio-Rad, Hercules, CA). Real-time analysis was performed on a CFX96 Touch System (Bio-Rad), and for the relative expression the Delta Delta Threshold Cycle (DDCt) method was used. Gene cluster analyses were performed with CFX Manager software 3.1 (Bio-Rad).

**Colon Cytokine Analysis**

Colons were explanted from experimental mice after being killed, then cut, opened, and washed from feces in an antibiotic solution. They then were minced in small pieces (approximately 0.5-cm long) and cultured overnight in RPMI 1640 (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific) and 100 U/mL penicillin/streptomycin (Thermo Fisher Scientific) at 37°C in a humidified 5% CO2 atmosphere. Supernatants were harvested 24 hours later and used for ELISA.

**Feces Analyses**

Stool samples of mice were collected on day 0 (before the first IP injection) and day 14 (corresponding to the end of treatment with vehicle or anakinra), and thereafter stored
Figure 11. (A) Representative density plot analysis of intracellular staining of IFN-γ from CD8+ T cells in LP of Winnie and Winnie-TNF-KO mice, treated with vehicle or anakinra. (B) Frequencies of IFN-γ+ from CD4+ and CD8+ T cells in the LP of Winnie and Winnie-TNF-KO mice treated with anakinra or vehicle. *P < .05, **P < .01.
at -80°C. For analysis, frozen feces were diluted in DPBS + 0.1% Tween 20 to a final concentration of 100 mg/mL, homogenized with tissue lyser for 5’ at 30 Hz, then centrifuged at 14,000 × g for 10 minutes at 4°C. The supernatants then were collected and stored at -80°C for ELISA.

**Generation and Culture of Murine DCs**

BMDCs were obtained from WT or TNF-KO mice. Single-cell suspension of BMDCs from the tibiae and femurs of 8- to 10-week-old male mice were flushed with 0.5 mmol/L EDTA (Thermo Fisher Scientific), and depleted of red blood cells with ACK lysing buffer (Thermo Fisher Scientific). Cells were plated in a 10-mL dish (1 × 10⁶ cells/mL) in RPMI 1640 (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific), 100 U/mL penicillin/streptomycin (Thermo Fisher Scientific), 25 ng/mL mouse recombinant GM-CSF (rmGM-CSF) and 25 ng/mL rmIL4 (Miltenyi Biotec) at 37°C in a humidified 5% CO₂ atmosphere. Five days after the isolation, all nonadherent cells were gently harvested and plated on a 24-well culture plate at a concentration of 1 × 10⁶ cells/mL, and new growth factors were added to the culture medium too. On day 7 cells were stimulated with 1 μg/mL LPS (L6143; Sigma-Aldrich). Supernatants were collected 24 hours after LPS stimulation.

**Multiplex Cytokine and Chemokine ELISA**

Cell culture supernatants were analyzed by multiplex to evaluate cytokine and chemokine concentrations, using the Bead-based Multiplex for the Luminex platform (LaboSpace srl, Milan, Italy).

**Study Population**

A total of 30 UC patients with moderate-to-severe disease starting IFX therapy were enrolled at the IBD Unit of Fondazione Policlinico Universitario “A. Gemelli” IRCCS, and the IBD Unit of IRCCS “S. De Bellis.” The general and clinical characteristics of the study population are detailed in Table 5. Patients were evaluated endoscopically at the beginning of anti-TNF treatment (T0) and the end of the induction regimen, specifically 12 weeks after the first infusion (T1). All subjects were naive to anti-TNF therapy and had stopped other immunosuppressant drugs at least 1 week before enrollment. The exclusion criteria were participants with diagnoses of comorbidities, such as diabetes, autoimmune disease, or any associated inflammatory or infectious disease. There were no significant differences among patients in risk factors and/or clinical variables such as sex, family history of IBD, tobacco smoking, body mass index, hemoglobin, glucose, triglycerides, total cholesterol, high-density lipoprotein, low-density lipoprotein, very-low-density lipoprotein, or systolic/diastolic blood pressure.

Serum samples were obtained from each UC patient at T0 and T1, and directly frozen at -80°C. The UC endoscopic classification was established using the endoscopic Mayo score of severity. At T0 all subjects had an endoscopic Mayo score ≥2. UC patients then were divided into 2 groups based on the endoscopic score at T1: endoscopic Mayo score of 0–1 (responders) and endoscopic Mayo score of ≥2 (nonresponders).

All enrolled subjects provided written informed consent.

**ELISA**

Sera from UC patients treated with IFX were analyzed for IL1β release in triplicate, using an ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturer’s instructions.

Supernatants from mice colonic tissue culture were analyzed for IL1β, IFNγ, IL6, and IL18 release in triplicate, using an ELISA kit (R&D Systems) following the manufacturer’s instructions.

Supernatants from mice stool were analyzed for N-GAL (lipocalin-2) release in triplicate, using an ELISA kit (R&D Systems) following the manufacturer’s instructions.

**Multiplex Cytokine Analysis With Fluorescence-Activated Cell Sorting**

Sera obtained from UC patients treated with IFX were analyzed using the MACSPlex Cytokine 12 Kit human (Miltenyi Biotec), following the manufacturer’s instructions. Flow cytometer acquisition was performed using NAVIOS (Beckman Coulter). Flow cytometer analysis was performed using Kaluza Software 1.5 (Beckman Coulter).

**Multiple Regression and Correlation Analysis**

The goodness-of-fit test and multiple regression were used to examine IL1β associations with other cytokines and chemokines in sera and to evaluate the correlation between the percentage of IL1β+ or caspase 8+ cells and inflammatory score in biopsy specimens. Correlation analysis was conducted using the Pearson test (r, regression). Results were considered statistically significant at P < .05.

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism (San Diego, CA) statistical software release 8.0. All data were expressed as means ± SEM obtained from at least 3 independent experiments. We evaluated statistical significance with a 2-tailed Student t test, 1-way analysis of variance followed by Tukey multiple comparison as post-test, and the 2-way analysis of variance test using the Bonferroni as a post-test for the grouped analysis.

### Table 5. General and Clinical Characteristics of Patients With UC Enrolled in the Analysis

| Patients, n | Disease | Male/female | Age, y | Montreal classification |
|------------|---------|-------------|--------|-------------------------|
| 30         | UC      | 17/13       | 43.5 ± 6.8 | 7 E2S2, 4 E2S3, 19 E3S2 |

---

**Flow Cytometer Acquisition**

Flow cytometer acquisition was performed using NAVIOS (Beckman Coulter). Flow cytometer analysis was performed using Kaluza Software 1.5 (Beckman Coulter).
Results were considered statistically significant at \( P < .05 \).

Access to Data

All authors had access to the study data and reviewed and approved the final manuscript.

References

1. Murch SH, Lamkin VA, Savage MO, Walker-Smith JA, MacDonald TT. Serum concentrations of tumour necrosis factor alpha in childhood chronic inflammatory bowel disease. Gut 1991;32:913.

2. Tsukada Y, Nakamura T, limura M, iizuka BE, Hayashi N. Cytokine profile in colonic mucosa of ulcerative colitis correlates with disease activity and response to granulocytapheresis. Am J Gastroenterol 2002;97:2820–2828.

3. Braegger CP, Nicholls S, Murch SH, Stephens S, MacDonald TT. Tumour necrosis factor alpha in stool as a marker of intestinal inflammation. Lancet 1992;339:89–91.

4. Sherman M, Tsynman DN, Kim A, Arora J, Pietras T, Messing S, St Hilaire L, Yoon S, Decross A, Shah A, Sauberlann L. Sustained improvement in health-related quality of life measures in patients with inflammatory bowel disease receiving prolonged anti-tumor necrosis factor therapy. J Dig Dis 2014;15:174–179.

5. Pisani LF, Moriggi M, Gelf C, Vecchi M, Pastorelli L. Proteomic insights on the metabolism in inflammatory bowel disease. World J Gastroenterol 2020;26:696–705.

6. Ben-Horin S, Kopylov U, Chowers Y. Optimizing anti-TNF treatments in inflammatory bowel disease. Autoimmun Rev 2014;13:24–30.

7. Lopetuso LR, Gerardi V, Papa V, Scaldaferrri F, Rapaccini GL, Gasbarrini A, Papa A. Can we predict the efficacy of anti-TNF-alpha agents? Int J Mol Sci 2017;18:1973.

8. Lopetuso LR, Gasbarrini A. Fighting the hype for predictors of efficacy in inflammatory bowel disease. Inflamm Bowel Dis 2020;26:764–765.

9. Kennedy NA, Heap GA, Green HD, Hamilton B, Bewshea C, Walker GJ, Thomas A, Nice R, Perry MH, Bouri S, Chanchlani N, Heerasing NM, Hendy P, Lin S, Gaya DR, Cummings JRF, Selinger CP, Lees CW, Hart AL, Parkes M, Sebastian S, Mansfield JC, Irving PM, Lindsay J, Russell RK, McDonald TJ, McGovern D, Goodhand JR, Ahmad T. Predictors of anti-TNF treatment failure in anti-TNF-naive patients with active luminal Crohn’s disease: a prospective, multicentre, cohort study. Lancet Gastroenterol Hepatol 2019;4:341–353.

10. Sprakes MB, Ford AC, Warren L, Greer D, Hamlin J. Efficacy, tolerability, and predictors of response to infliximab therapy for Crohn’s disease: a large single centre experience. J Crohns Colitis 2012;6:143–153.

11. Wong U, Cross RK. Primary and secondary nonresponse to infliximab: mechanisms and countermeasures. Expert Opin Drug Metab Toxicol 2017;13:1039–1046.

12. West NR, Hegazy AN, Owens BMJ, Bullers SJ, Linggi B, Buonocore S, Coccia M, Geïordt D, Thiss S, Stockenhuber K, Pott J, Friedrich M, Ryzhakov G, Barb全体员工 C, Brodmelt CR, Cieluch C, Rahman N, Müller-Növen G, Owens RJ, Kühl AA, Maloy KJ, Plevy SE, Keshaw S, Travis SPL, Powrie F, Oncostatin M drives intestinal inflammation and predicts response to tumour necrosis factor-neutralizing therapy in patients with inflammatory bowel disease. Nat Med 2017;23:579–589.

13. Dinarello CA, Simon A, van der Meer JW. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. Nat Rev Drug Discov 2012;11:633–652.

14. Dinarello CA. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. Blood 2011;117:3720–3732.

15. Cominelli F, Pizarro TT. Interleukin-1 and interleukin-1 receptor antagonist in inflammatory bowel disease. Aliment Pharmacol Ther 1996;10(Suppl 2):49–53; discussion 4.

16. Dinarello CA. Overview of the IL-1 family in innate inflammation and acquired immunity. Immunol Rev 2018;281:8–27.

17. Dinarello CA, van der Meer JW. Treating inflammation by blocking interleukin-1 in humans. Semin Immunol 2013;25:469–484.

18. Cohen S, Hurd E, Cush J, Schiff M, Weinblatt ME, Moreland LW, Kremer J, Bear MB, Rich WJ, McCabe D. Treatment of rheumatoid arthritis with anakinra, a recombinant human interleukin-1 receptor antagonist, in combination with methotrexate: results of a twenty-four-week, multicenter, randomized, double-blind, placebo-controlled trial. Arthritis Rheum 2002;46:614–624.

19. Cohen SB, Moreland LW, Cush JJ, Greenwald MW, Block S, Shergy WJ, Hanrhaner PS, Kraishi MM, Patel A, Sun G, Bear MB. A multicentre, double blind, randomised, placebo controlled trial of anakinra (Kinereet), a recombinant interleukin 1 receptor antagonist, in patients with rheumatoid arthritis treated with background methotrexate. Ann Rheum Dis 2004;63:1062–1068.

20. Bresnihan B, Alvaro-Gracia JM, Cobby M, Doherty M, Domljjan I, Emery P, Nuki G, Pavelka K, Rau R, Rozman B, Watt I, Williams B, Aitchison R, McCabe D, Musiak P. Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. Arthritis Rheum 1998;41:2196–2204.

21. Opal SM, Fisher CJ Jr, Dhainaut JF, Vincent JL, Brase R, Lowry SF, Sadoff JC, Slotman GJ, Levy H, Balk RA, Shelly MP, Pribble JP, LaBrecque JF, Lookabaugh J, Donovan H, Dubin H, Baughman R, Norman J, DeMaria E, Matzel K, Abraham E, Seneff M. Confirmatory interleukin-1 receptor antagonist trial in severe sepsis: a phase III, randomized, double-blind, placebo-controlled, multicentre trial. The Interleukin-1 Receptor Antagonist Sepsis Investigator Group. Crit Care Med 1997;25:1115–1124.

22. Aschenbrenner D, Quantra M, Banerjee S, Iloot N, Janssen J, Steere B, Chen YH, Ho S, Cox K, Arancibia-Cárncamo CV, Coles M, Gaffney E, Travis SP, Denson L, Kugathasan S, Schmitz J, Powrie F, Sansom SN, Uhlig HH. Deconvolution of monocyte responses in...
inflammatory bowel disease reveals an IL-1 cytokine network that regulates IL-23 in genetic and acquired IL-10 resistance. Gut 2021;70:1023–1036.

23. McGovern D, Powrie F. The IL23 axis plays a key role in the pathogenesis of IBD. Gut 2007;56:1333–1336.

24. De Santis S, Kunde D, Galleggiante V, Liso M, Scandiffo L, Serino G, Pinto A, Campiglia P, Sorrentino R, Cavalcanti E, Santino A, Caruso ML, Eri R, Chieppa M. TNFα deficiency results in increased IL-1β in an early onset of spontaneous murine colitis. Cell Death Dis 2017;8:e2993.

25. Eri RD, Adams RJ, Tran TV, Tong H, Das I, Roche DK, Oancea I, Png CW, Jeffery PL, Radford-Smith GL, Cook MC, Florin TH, McGuckin MA. An intestinal epithelial defect conferring ER stress results in inflammation involving both innate and adaptive immunity. Mucosal Immunol 2011;4:354–364.

26. Liso M, De Santis S, Verna G, Dicarlo M, Calasso M, Santino A, Gigante I, Eri R, Raveenthiraraj S, Sobolewski A, Palmietta V, Lippolis A, Mastronardi M, Armentano R, Serino G, De Angelis M, Chieppa M. A specific mutation in Muc2 determines early dysbiosis in colitis-prone Winnie mice. Inflamm Bowel Dis 2020;26:546–556.

27. Chen L, Zhou Z, Yang Y, Chen N, Xiang H. Therapeutic effect of imiquimod on dextran sulfate sodium-induced ulcerative colitis in mice. PLoS One 2017;12:e0186138.

28. Stokkers PC, van Aken BE, Basoski N, Reitsma PH, Tytgat GN, van Deventer SJ. Five genetic markers in the interleukin 1 family in relation to inflammatory bowel disease. Gut 1998;43:33–39.

29. Heresbach D, Alizadeh M, Dabadie A, Le Berre N, Colombel JF, Yaqouan J, Bretagne JF, Semana G. Significance of interleukin-1β and interleukin-1 receptor antagonist genetic polymorphism in inflammatory bowel diseases. Am J Gastroenterol 1997;92:1164–1169.

30. Thomas MG, Bayliss C, Bond S, Dowling F, Galea J, Jairath V, Lamb C, Probert C, Timperley-Preece E, Watson A, Whitehead L, Williams JG, Parkes M, Kaser A, Raine T. Trial summary and protocol for a phase II randomised placebo-controlled double-blinded trial of Interleukin 1 blockade in Acute Severe Colitis: the IASO trial. BMJ Open 2019;9:e023765.

31. Kruiding M, Evan GI. Caspase-8 in apoptosis: the beginning of “the end”? IUBMB Life 2000;50:85–90.

32. Schwarzr H, Jiao H, Wachsmuth L, Tresch A, Pasparakis M. FADD and caspase-8 regulate gut homeostasis and inflammation by controlling MLKL- and GSDMD-mediated death of intestinal epithelial cells. Immunity 2020;52:978–993.e6.

33. Prokhorova EA, Kopeina GS, Lavrik IN, Zhivotovsky B. Apoptosis regulation by subcellular relocation of caspases. Sci Rep 2018;8:12199.

34. Benchoua A, Couriaud C, Guégan C, Tartier L, Couvert P, Friocourt G, Chelly J, Méniassier-de Murcia J, Onténiente B. Active caspase-8 translocates into the nucleus of apoptotic cells to inactivate poly(ADP-ribose) polymerase-2. J Biol Chem 2002;277:34217–34222.

35. Feltham R, Vince JE, Lawlor KE. Caspase-8: not so silently deadly. Clin Transl Immunol 2017;6:e124.

36. Gurung P, Kanneganti TD. Novel roles for caspase-8 in IL-1β and inflammasome regulation. Am J Pathol 2015;185:17–25.

37. Monie TP, Bryant CE. Caspase-8 functions as a key mediator of inflammation and pro-IL-1β processing via both canonical and non-canonical pathways. Immunol Rev 2015;265:181–193.

38. Shohan M, Sabzevaray-Ghaftarokhi M, Bagheri N, Shirzad H, Rahimian G, Soltani A, Ghatreh-Samani M, Deris F, Tahmasbi K, Shahverdi E, Fathollahi F. Intensified Th9 response is associated with the immunopathogenesis of active ulcerative colitis. Immunol Invest 2018;47:700–711.

39. Corridoni D, Antanaviciute A, Gupta T, Fawkner-Corbett D, Aulicino A, Jagielowicz M, Parikh K, Repapi E, Taylor S, Ishikawa D, Hatano R, Yamada T, Xin W, Slawinski H, Bowden R, Napolitani G, Brain O, Morimoto C, Koohy H, Simmons A. Single-cell atlas of colonic CD8+ T cells in ulcerative colitis. Nat Med 2020;26:1480–1490.

40. Larochette V, Miot C, Poli C, Beaumont E, Roingeard P, Fickenscher H, Jeannin P, Delneste Y. IL-26, a cytokine with roles in extracellular DNA-induced inflammation and microbial defense. Front Immunol 2019;10:204.

41. Yang XP, Albrecht U. Dual function of interleukin-1beta for the regulation of interleukin-6-induced suppressor of cytokine signaling 3 expression. J Biol Chem 2004 Oct 22;279(43):45279–45289.

42. Obici L, Meini A, Catalini M, Chicca S, Galliani M, Donadel S, Plebani A, Merlini G. Favourable and sustained response to anakinra in tumour necrosis factor receptor-associated periodic syndrome (TRAPS) with or without AA amyloidosis. Ann Rheum Dis 2011;70:1511–1512.

43. Sacré K, Brihaye B, Lidove O, Papo T, Pocidalo MA, Cuisset L, Dodé C. Dramatic improvement following interleukin 1beta blockade in tumor necrosis factor receptor-1-associated syndrome (TRAPS) resistant to anti-TNF-alpha therapy. J Rheumatol 2008;35:357–358.

44. Lobito AA, Kimberley FC, Muppidi JR, Komarow H, Jackson AJ, Hull KM, Kastner DL, Scroaton GR, Siegel RM. Abnormal disulphide-linked oligomerization results in ER retention and altered signaling by TNFR1 mutants in TNFR1-associated periodic fever syndrome (TRAPS). Blood 2006;108:1320–1327.

45. Simon A, Park H, Maddipati R, Lobito AA, Bulau AC, Jackson AJ, Chae JJ, Ettinger R, de Koning HD, Cruz AC, Kastner DL, Scroaton GR, Siegel RM. Concerted action of wild-type and mutant TNF receptors enhances inflammation in TNF receptor 1-associated periodic fever syndrome. Proc Natl Acad Sci U S A 2010;107:9801.

46. Lim W-K, Fujimoto C, Ursea R, Mahesh SP, Silver P, Chan C-C, Gery I, Nussenblatt RB. Suppression of immune-mediated ocular inflammation in mice by interleukin 1 receptor antagonist administration. Arch Ophthalmol 2005;123:957–963.

47. Abbate A, Salloum FN, Vecile E, Das A, Hoke NN, Straino S, Biondi-Zoccai GGL, Houser J-E, Qureshi IZ, Ownby ED, GSTinini E, Biasucci LM, Severino A, Capogrossi MC, Vetrovec GW, Crea F, Baldi A, Kukreja RC, Dobrina A. Anakinra, a recombinant human
interleukin-1 receptor antagonist, inhibits apoptosis in experimental acute myocardial infarction. Circulation 2008;117:2670–2683.

48. D’Haens G, Sandborn WJ, Feagan BG, Geboes K, Hanauer SB, Irvine EJ, Lémann M, Marteau P, Rutgeerts P, Schölmerich J, Sutherland LR. A review of activity indices and efficacy end points for clinical trials of medical therapy in adults with ulcerative colitis. Gastroenterology 2007;132:763–786.