Blockade of cytotoxic T-lymphocyte antigen-4 by ipilimumab results in dysregulation of gastrointestinal immunity in patients with advanced melanoma

David Berman, Susan M. Parker, Jonathan Siegel, Scott D. Chasalow, Jeffrey Weber, Susan Galbraith, Stephan R. Targan and Hanlin L. Wang

1Bristol-Myers Squibb Company, Princeton, New Jersey, USA
2Bristol-Myers Squibb Company, Wallingford, Connecticut, USA
3Comprehensive Melanoma Research Center, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida, USA
4Division of Gastroenterology, Cedars-Sinai Medical Center, Los Angeles, California, USA
5Department of Pathology and Laboratory Medicine, Cedars-Sinai Medical Center, Los Angeles, California, USA

Communicated by: LJ Old

Blockade of cytotoxic T-lymphocyte antigen-4 (CTLA-4) by ipilimumab leads to immune-mediated tumor regression and immune-related adverse events (irAEs), including diarrhea and colitis. The current analyses were undertaken to promote an understanding of the underlying mechanism of action and to identify potential biomarkers that could help in the prediction and management of ipilimumab-induced gastrointestinal irAEs. Treatment-naïve or previously treated patients with unresectable stage III/IV melanoma (n = 115) received open-label ipilimumab (10 mg/kg every 3 weeks for four doses) and were randomized to receive concomitant blinded prophylactic oral budesonide (9 mg/d with gradual taper through week 16) or placebo. Outcome measures included histologic assessment of bowel biopsies and assessment of serologic markers of inflammatory bowel disease (IBD), fecal calprotectin levels, and polymorphisms in immune-related genes. Ipilimumab resulted in dysregulation of gastrointestinal mucosal immunity as evidenced by altered antibody levels to enteric flora, inflammatory cell infiltration into gastrointestinal mucosa, and increased fecal calprotectin associated with diarrhea and clinical evidence of colitis. The pattern of ipilimumab-induced antibody titers to microbial flora and the histologic features and location of the inflammation were distinct from classic IBD. Prophylactic budesonide did not prevent ipilimumab-induced bowel inflammation. Despite an observed association between colonic inflammation and grade 2 or higher diarrhea, no baseline biomarkers could reliably predict development of gastrointestinal toxicity. Although classic IBD and ipilimumab-related gastrointestinal toxicity are both immune mediated, the observed pattern of biomarkers suggests ipilimumab-related gastrointestinal toxicity may be a distinct clinicopathologic entity.

Keywords: human, melanoma, ipilimumab, CTLA-4, gastrointestinal, immune-related adverse event

Introduction

Ipilimumab is a fully human monoclonal antibody directed against cytotoxic T-lymphocyte antigen-4 (CTLA-4), a key negative regulator of T-cell activation (1-3). Data from a phase II trial of treatment-naïve and previously treated patients with melanoma demonstrate tumor regression and encouraging median overall survival and 2-yr survival rates (18-19 mo, 41%-42%, respectively) with 10 mg/kg ipilimumab administered on a 3-weekly schedule (4). In a recent phase III trial, 3 mg/kg ipilimumab monotherapy demonstrated a statistically significant improvement in overall survival with 2 year survival rates of >23% in previously treated patients with advanced melanoma (5).

Genetic knock-out of CTLA-4 in mice results in diffuse infiltration of inflammatory immune cells into multiple organs due to peripheral T-cell proliferation (6). Not surprisingly, blockade of CTLA-4 by monoclonal antibodies results in immune-related adverse events (irAEs), including diarrhea and colitis (3). Examination of colonic biopsies obtained after onset of diarrhea or colitis reveals both acute and chronic inflammation (7). However, these biopsies were obtained after the onset of significant diarrhea, and no other characterization of gastrointestinal (GI) mucosal immunity has been examined. The etiology of classic inflammatory bowel disease (IBD), such as Crohn’s disease (CD) and ulcerative colitis (UC), results from dysregulated GI mucosal immunity, possibly related to both genetic susceptibility and an environmental component not yet known but suggested to be related to commensal bacteria (8-10). Thus, there is interest in exploring the relationship between GI irAEs resulting from CTLA-4 blockade and classic IBD.

The current analyses were undertaken to promote an understanding of the underlying mechanism of action and to identify potential biomarkers that could help in the prediction and management of ipilimumab-induced GI irAEs. These analyses were part of CA184-007 / NCT00135408, a randomized, controlled phase II study in patients with unresectable stage III/IV melanoma receiving open-label ipilimumab with concomitant prophylactic budesonide or placebo. The full clinical results of this trial, including the primary endpoint, are reported elsewhere (4).
Table 1

Frequency of grade 2 or higher diarrhea or colitis by endoscopic and microscopic findings after first dose of ipilimumab.

<table>
<thead>
<tr>
<th>Endoscopic signs of colitis</th>
<th>Ipilimumab + budesonide</th>
<th>Ipilimumab + placebo</th>
<th>Ipilimumab + budesonide</th>
<th>Ipilimumab + placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients in subset, n/N (%)</td>
<td>10/53 (18.9)</td>
<td>14/53 (26.4)</td>
<td>43/53 (81.1)</td>
<td>39/53 (73.6)</td>
</tr>
<tr>
<td>Rate of grade ≥2 diarrhea or colitis before week 24, n/N (%)</td>
<td>5/10 (50.0)</td>
<td>5/14 (35.7)</td>
<td>14/43 (32.6)</td>
<td>13/39 (33.3)</td>
</tr>
<tr>
<td>95% CI for rate</td>
<td>18.7, 81.3</td>
<td>12.8, 64.9</td>
<td>19.1, 46.5</td>
<td>19.1, 50.2</td>
</tr>
</tbody>
</table>

Pre-specified microscopic findings

| Patients in subset, n/N (%) | 8/45 (17.8) | 6/37 (16.2) | 37/45 (82.2) | 31/37 (83.8) |
| Rate of grade ≥2 diarrhea or colitis before week 24, n/N (%) | 7/9 (75.0) | 4/6 (66.7) | 11/36 (30.6) | 7/31 (22.6) |
| 95% CI for rate | 34.9, 96.8 | 22.3, 95.7 | 18.0, 49.8 | 9.6, 41.1 |

Results

Patient characteristics

In total, 135 patients were enrolled and 115 were randomized. All randomized patients were treated and received both ipilimumab and blinded oral study medication. Demographics and other baseline characteristics have been reported previously (4).

Clinical characteristics of ipilimumab-induced GI toxicity

Prophylactic budesonide did not prevent the onset of GI irAEs or have an effect on any of the biomarker analyses listed below. For ipilimumab plus budesonide (budesonide group) and ipilimumab plus placebo (placebo group), 19 (32.8%) of 58 and 20 (35.1%) of 57 patients, respectively, had grade 2 or higher diarrhea during induction. Grade 2 or higher colitis during induction was reported in eight (13.8%) of 58 and seven (12.3%) of 57 patients in the budesonide and placebo groups, respectively. Incidence of diarrhea and colitis overlapped in some patients. Thus in total, 21 (36.2%) of 58 patients in the budesonide group and 20 (35.1%) of 57 patients in the placebo group experienced grade 2 or higher diarrhea and/or grade 2 or higher colitis during the 23 week induction period, the time period used for the primary analysis. Grade 2 or higher diarrhea or colitis occurred at least once during the study in 22 (37.9%) of 58 and 21 (36.8%) of 57 patients in the budesonide and placebo groups, respectively.

Onset of grade 2 or higher GI irAEs generally occurred in the first 16 weeks of treatment, and most GI irAEs were reversible using product-specific treatment guidelines including vigilant follow-up and early use of steroids when appropriate. Three patients had grade 2 or higher GI irAEs within the first 2 weeks. No patient reported GI perforation or required colectomy. During the induction period, the median time to resolution (to grade 1 or less) of grade 2-4 diarrhea or clinical colitis regardless of causality was 3.4 weeks (95% confidence interval [CI]: 0.7-4.3) and 2.0 weeks (95% CI: 1.4-3.4) in the budesonide and placebo groups, respectively. Among the 14 patients in the budesonide group and 13 patients in the placebo group who experienced severe (grade 3-4) GI irAEs during induction, the median time to resolution was 2.8 weeks (95% CI: 0.7-7.0) and 1.9 weeks (95% CI: 0.6-2.9), respectively. The majority of these patients, 9 and 10 patients, respectively, received systemic immunosuppression. The longest time to resolution for any single grade 2-4 GI irAE was approximately 18 weeks, which occurred in a patient receiving systemic immunosuppression.

GI endoscopy and histopathology in patients after the first dose of ipilimumab

Protocol-specific endoscopy and biopsy were performed 1 to 2 weeks after the first dose of ipilimumab to characterize incipient induced inflammatory changes in the bowel mucosa before potential secondary, non-specific ulceration and inflammatory changes occurred. Lower GI endoscopy was reported between day 1 and 15 in 53 (91.4%) of 58 and 53 (93.0%) of 57 patients in the budesonide group and placebo groups, respectively, with the rectum and sigmoid colon being the most common sites of evaluation (approximately 85% of all treated patients in both groups).

No association between any abnormal early endoscopic finding and development of grade 2 or higher diarrhea was observed. Among 10 patients in the budesonide group and 14 in the placebo group with at least one abnormal early endoscopic finding, five patients in each group (50.0% and 35.7%, respectively) had grade 2 or higher diarrhea/clinical colitis before week 24 (Table 1). Among 43 patients in the budesonide group and 39 in the placebo group with no abnormal endoscopic findings, 14 (32.6%) and 13 (33.3%), respectively, had grade 2 or higher diarrhea/clinical colitis before week 24. One patient receiving budesonide had grade 2 diarrhea before the date of endoscopy but had no abnormal endoscopic findings. Evaluable colonic biopsies were collected from 45 (77.6%) of 58 and 37 (64.9%) of 57 patients in the budesonide and placebo group, respectively. Sites were instructed to obtain biopsies of any lesions; or if none, then five sites of the investigators’ choice. The most frequent biopsy sites were the sigmoid colon (38 [65.5%] of 58 patients in the budesonide group and 29 [50.9%] of 57 patients in the placebo group), rectum (33 [56.9%] of 58 patients in the budesonide group and 19 [33.3%] of 57 patients in the placebo group), and the left colon (13 [22.4%] of 58 patients in the budesonide group and nine [15.8%] of 57 patients in the placebo group) (Table 2).

Hematoxylin and eosin (H&E)-stained sections of the biopsies were examined in a blinded manner for a prospectively defined list of histologic findings considered to be abnormal in routine biopsy of a healthy person. Across all evaluable patients and all
biopsies following the first dose of ipilimumab. Histopathologic
appeared more severe than those observed in the pre-specified
evaluated, as full endoscopies were not performed.
from the distal colon. Pathology in the proximal colon was not
appeared to be inflamed by endoscopy. All of these biopsies were
and biopsy, respectively. Biopsies were obtained from sites that
higher diarrhea before week 24 underwent repeat endoscopy
diarrhea. Biopsy of this patient (5 d after first onset of diarrhea)
colitis before biopsy; this patient had prior grade 2 or higher
diarrhea or clinical colitis during induction (Table 1).
A trend toward the presence of at least one histologic finding
and subsequent development of grade 2 or higher diarrhea/
clinical colitis was suggested. Among the nine patients in the
budesonide group and six patients in the placebo group with at
least one of these inflammatory markers, seven (77.8%) patients
and four (66.7%) patients, respectively, had grade 2 or higher
diarrhea or clinical colitis during induction. Among the 36
patients in the budesonide group and 31 patients in the placebo
group with none of these pre-specified markers, 11 (30.6%)
patients and 7 (22.6%) patients, respectively, had grade 2 or
higher diarrhea or clinical colitis during induction (Table 1).
For all but one patient, histologic findings preceded the onset
of grade 2 or higher diarrhea or colitis. Only one patient with at
least one of these histologic findings had any-grade diarrhea or
colitis before biopsy; this patient had prior grade 2 or higher
diarrhea. Biopsy of this patient (5 d after first onset of diarrhea)
included focal eosinophilic and neutrophilic cryptitis, focal
lamina propria neutrophilic infiltration, and focal mucin
depletion. All other patients with at least one microscopic
finding had a temporal separation with subsequent grade 2 or
higher diarrhea; the majority exhibiting at least 3 weeks between
biopsy and the diarrhea.

**VI histopathology in patient biopsies after onset of grade 2 or higher
diarrhea**

Only 17 of 39 and 10 of 39 patients who developed grade 2 or
higher diarrhea before week 24 underwent repeat endoscopy
and biopsy, respectively. Biopsies were obtained from sites that
appeared to be inflamed by endoscopy. All of these biopsies were
from the distal colon. Pathology in the proximal colon was not
evaluated, as full endoscopies were not performed.

Changes in the bowel mucosa following onset of diarrhea
appeared more severe than those observed in the pre-specified
biopsies following the first dose of ipilimumab. Histopathologic
examination of the biopsies revealed active colitis characterized
by marked lamina propria mixed inflammatory cell infiltrates
consisting of neutrophils, lymphocytes, plasma cells, and
eosinophils (Figure 1A). Foci of neutrophilic cryptitis (Figure 1B),
crypt abscesses (Figure 1C), glandular destruction, and erosions of the mucosal surface were evident. Ulceration
was noted occasionally. Inflammatory changes were diffuse in
75% of the biopsies and patchy in the remaining cases (Figure 1D). There was no meaningful increase in the number of
intraepithelial lymphocytes or apoptotic activity in colonocytes.
Histologic evidence of chronicity, such as crypt architectural
distortion, basal plasmacytosis, granuloma, Paneth cell
metaplasia, or pyloric metaplasia, was not evident.

**Ipilimumab induced fluctuations in antibodies to enteric flora**

In normal healthy people, the humoral response to enteric
flora is maintained in homeostasis. Perturbation of this
homeostasis, manifested as increasing antibody levels to select
enteric flora, is characteristic of IBD but not acute GI
inflammation (i.e., diverticulitis/infection) (8-10). Blockade of
CTLA-4 by ipilimumab induced fluctuations in the levels of one
or more of those antibodies in given patients (Figure 2 and
Figure 3). The levels of antibody could exhibit increases,
decreases, or both during the induction phase with similar
degrees of fluctuation for all studied antibodies (representative
changes for *Pseudomonas* anti-I2 are shown in Figure 2).
Patients with higher mean antibody levels had greater standard
deviations than those with lower mean antibody levels. In
subjects with grade 2 or higher GI irAEs, the most common
positive antibody titers were to the perinuclear-staining anti-
neutrophil cytoplasmic antibody (pANCA) and OmpC (*E. coli*)
(Table 3). No strong associations between a positive level and GI
irAEs were observed. Most subjects who were positive for anti-
I2, anti-*Saccharomyces cerevisiae* antibody (ASCA), or CBir
flagellin antibody (CBir1) did not have any grade GI irAE, while
approximately 50% of subjects positive for pANCA or OmpC
had at least a grade 1 GI irAE. In subjects with grade 2 or higher
GI irAEs, the highest frequency of patients with positive titers
were seen with anti-pANCA (21.4%) and anti-OmpC (40.5%),
with <10% of patients positive for anti-I2, anti-ASCA, and anti-
CBir1 (data not shown).

### Table 2

<table>
<thead>
<tr>
<th>Microscopic findings</th>
<th>Left Colon</th>
<th>Signoid</th>
<th>Rectum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ipilimumab+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>budesonide</td>
<td>placebo</td>
<td></td>
</tr>
<tr>
<td>Lymphocytic cryptitis</td>
<td>2/13 (15.4)</td>
<td>1/9 (11.1)</td>
<td>0/0 (0)</td>
</tr>
<tr>
<td>Lamina propria plasma cell excess</td>
<td>3/13 (23.1)</td>
<td>2/9 (22.2)</td>
<td>3/8 (38.1)</td>
</tr>
<tr>
<td>Lamina propria neutrophilic infiltration</td>
<td>3/13 (23.1)</td>
<td>0/0 (0)</td>
<td>5/38 (13.2)</td>
</tr>
</tbody>
</table>

Three most frequent microscopic findings in gastrointestinal biopsies after first dose of ipilimumab, by location.
**Figure 1**

![Histopathologic examination of GI biopsies.](image)

Representative hematoxylin and eosin (H&E) section demonstrating active colitis characterized by marked lamina propria mixed inflammatory cell infiltrates consisting of neutrophils, lymphocytes, plasma cells, and eosinophils (A). Foci of neutrophilic cryptitis (B) and crypt abscesses (C) were also evident. For the majority (75%) of patients, inflammatory changes were diffuse, with the remainder being patchy in nature (D).

**Figure 2**

![Fluctuating levels of anti-I2 antibodies in serum from patients with grade 0-1 GI irAEs.](image)

Fluctuating levels of anti-I2 antibodies in serum from patients with grade 0-1 GI irAEs. Well-characterized IBD markers, including antibodies to microbial antigens, were evaluated by ELISA using serum samples taken at baseline and throughout the induction phase. Data for the *Pseudomonas* I2 antigen from both the budesonide-treated and placebo-treated groups are illustrated. The x value for each point is the mean of the multiple time points for a subject; the y value is the standard deviation of the multiple time points for a subject. Large y values represent large fluctuations over time within the subject. The data are representative of results obtained for other microbial antigens and other GI irAE grades.
Ipilimumab results in dysregulation of mucosal immunity. Plots of the time course of individual fecal calprotectin levels overlaid with onset dates of GI (G), skin (S), hepatic (H), and any other (O) irAEs are shown for representative patients in the left panel. Inverted black triangles indicate dosing dates. Plotting symbols (1 through 5) indicate grade of irAE. Vertical lines: black = date of first dose; gray = date of GI irAE onset. These data illustrate that elevated fecal calprotectin levels, relative to baseline, do not appear specific to patients reporting a grade 2 or higher irAE. The panel on the right illustrates, for representative patients, changes over time in titers of antibodies to enteric flora (ASCA, I2, CBir1, OmpC, and pANCA).

Table 3
Frequency of worst-grade GI irAE by IBD marker and biomarker status across all patients.

<table>
<thead>
<tr>
<th>GI irAEs</th>
<th>Anti-I2</th>
<th>Anti-ASCA IgA</th>
<th>Anti-ASCA IgG</th>
<th>Anti-CBir1</th>
<th>Anti-pANCA</th>
<th>Anti-OmpC</th>
</tr>
</thead>
<tbody>
<tr>
<td>None, n</td>
<td>13</td>
<td>48</td>
<td>11</td>
<td>50</td>
<td>13</td>
<td>48</td>
</tr>
<tr>
<td>(%)</td>
<td>(72.2)</td>
<td>(49.5)</td>
<td>(64.7)</td>
<td>(51.0)</td>
<td>(72.2)</td>
<td>(49.5)</td>
</tr>
<tr>
<td>Grade 1, n</td>
<td>2</td>
<td>10</td>
<td>2</td>
<td>10</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>(%)</td>
<td>(11.1)</td>
<td>(10.3)</td>
<td>(11.8)</td>
<td>(10.2)</td>
<td>(11.1)</td>
<td>(10.3)</td>
</tr>
<tr>
<td>Grade ≥2, n</td>
<td>3</td>
<td>38</td>
<td>4</td>
<td>39</td>
<td>4</td>
<td>38</td>
</tr>
<tr>
<td>(%)</td>
<td>(16.7)</td>
<td>(40.2)</td>
<td>(23.5)</td>
<td>(36.8)</td>
<td>(16.7)</td>
<td>(40.2)</td>
</tr>
<tr>
<td>Total, n</td>
<td>18</td>
<td>97</td>
<td>17</td>
<td>98</td>
<td>18</td>
<td>97</td>
</tr>
<tr>
<td>(%)</td>
<td>(100)</td>
<td>(100)</td>
<td>(100)</td>
<td>(100)</td>
<td>(100)</td>
<td>(100)</td>
</tr>
</tbody>
</table>

*The denominator for percentages is the total number of patients per biomarker status group for each IBD marker.

Abbreviations: GI, gastrointestinal; IBD, inflammatory bowel disease; irAE, immune-related adverse event.

Ipilimumab induced an increase in fecal calprotectin that was not specific for diarrhea or colitis

Fecal calprotectin, derived predominantly from neutrophils that migrate into the intestinal mucosa, is a biomarker of bowel inflammation (11). No meaningful differences in fecal calprotectin levels were observed between the two treatment groups in this trial (data not shown). The mean rate of change in calprotectin levels over the first 12 weeks after ipilimumab administration was 3.94 µg/g/day (95% CI: 1.21-6.67). This mean increase over time was statistically significant (F = 8.09; DF = 1267; P = 0.0048). Plots of the time course of individual fecal calprotectin levels overlaid with onset dates of GI, skin, hepatic, and any other irAEs showed that elevated fecal calprotectin level relative to baseline did not appear to be specific to patients reporting grade 2 or higher GI irAEs (Figure 3). However, as Figure 3 demonstrates, a rise in antibodies to CD- and UC-associated microbial antigens could parallel and in some cases even preceded the rise in fecal calprotectin.

Genetic polymorphisms did not predict GI toxicity

Association with worst-grade GI irAE was studied for 20 genetic polymorphisms in 10 immune-related genes. No
polymorphism exhibited a statistically significant departure from Hardy-Weinberg equilibrium. No association between genotype and worst-grade GI irAE was observed for any of the 18 polymorphisms analyzed (results not shown).

HLA-A and -B genotypes were available for 100 treated subjects. Fifteen unique HLA-A alleles and 22 unique HLA-B alleles were detected. Only five subjects were homozygous for HLA-A and none for HLA-B. Because many different HLA-A and HLA-B alleles were observed, analysis of allele pairs as co-dominant genotypes was impractical. Instead, possible associations with GI irAEs were analyzed for each allele separately. For HLA-A and -B, four and seven alleles, respectively, were carried by at least 10% of all treated subjects with HLA data. Only these “common” alleles were included in further analyses: A01, A02, A03, A24, B07, B08, B27, B35, B40, B44, and B57. No associations between HLA-A or HLA-B allele carrier status and worst-grade GI irAE were observed (results not shown).

Discussion

Blockade of CTLA-4 by ipilimumab in patients with melanoma most frequently results in GI and skin irAEs, sites that are exposed to the commensal flora (4). Results from this study indicate that blockade of CTLA-4 by ipilimumab was sufficient to cause dysregulation of GI mucosal immunity as evidenced by fluctuating antibody titers to enteric flora, increased levels of neutrophil-derived fecal calprotectin, and immune infiltration into the mucosa. However, the pattern of GI-specific biomarker changes is distinct from those observed for classic IBD.

Development of antibodies to enteric flora, including elevated titers of antibodies to key intestinal flora antigens, is a marker of a dysregulated mucosal immune environment in IBD but not acute (diverticulitis/infection) inflammation (8, 12). The pattern of antibody positivity to enteric flora observed in this study was not consistent with that for classic UC or CD. In CD, approximately half of patients are positive for ASCA, anti-CBir1, and anti-12, and <25% are positive for pANCA; in UC, approximately half of patients are positive for pANCA, but <10% are positive for ASCA, anti-CBir1, anti-12, and anti-OmpC (9, 11). In this study, the most common positive titers in patients with grade 2 or higher irAEs were to pANCA and anti-OmpC, with <10% of patients positive for anti-12 and ASCA, and <15% positive for anti-CBir1. Furthermore, ASCA or pANCA positivity is highly predictive for IBD (13), yet in the present study, half of patients with ASCA or pANCA positivity had no GI irAEs. Finally, the fluctuating antibody titers observed in the present study were also inconsistent with CD, where titers are stable over time and with change in disease activity (14). This fluctuation may reflect changes in the state of T-cell activation as ipilimumab concentrations cross an unidentified threshold. The parallel and transient changes in antibody levels in conjunction with an increase in fecal calprotectin are consistent with dysregulated mucosa induced by CTLA-4 blockade in these non-IBD patients.

The location and features of the bowel mucosa pathology observed in this study, or in an earlier reported study (7), are not characteristic of either CD or UC. The predominantly diffuse nature of the active inflammation in colonic biopsies from patients after onset of diarrhea or colitis are similar to UC, but features of chronicity and diffuse colonic involvement distally (hallmarks of UC) were not observed. Nor were the distinctive features of CD, including granulomas, aphthous or fissuring ulcers, and bowel wall thickening secondary to transmural inflammation observed in this study (10, 15-17). CD is primarily a disease of the proximal colon and terminal ileum whereas the majority of abnormal histologic findings in this study were located distally; although it should be noted that full colon endoscopy was not frequently performed in this study. Finally, the histologic findings observed here were also distinct from graft-vs.-host disease, which is characterized by prominent epithelial apoptosis and glandular destruction (18, 19).

Oral budesonide, used in the treatment of CD, did not prevent GI irAEs perhaps due to the majority of budesonide being delivered to the distal ileum and proximal colon, rather than the distal colon. Alternatively, prophylactically absorbed budesonide may not have been sufficient to suppress the immune-mediated diarrhea or colitis.

Biomarkers to reliably predict which patients would develop GI irAEs were not identified. Immune cell infiltration of the bowel mucosa early in treatment is suggested to be associated with later onset of diarrhea/colitis but is not reliable enough for routine use. No association between abnormal endoscopic findings and diarrhea/colitis were observed, possibly due to the lower sensitivity and lack of central review. Neutrophil-derived fecal calprotectin, a biomarker of active IBD (20, 21), increases upon ipilimumab treatment, indicating active inflammation in the bowel wall but cannot be used to predict onset of any irAE. No associations between GI irAEs and any of 18 single nucleotide polymorphisms (SNPs) in 10 immune-related genes were observed, despite previously reported association of CTLA-4 polymorphisms with autoimmune disease (22) and response to anti-CTLA-4 (23), NOD2 polymorphisms with IBD (24), CD86 (receptor for CTLA-4) polymorphisms with clinical allergic phenotype (25), and MHC class I polymorphisms with autoimmune diseases (26) and sensitivity to immunotherapy (27).

In conclusion, blockade of CTLA-4 by ipilimumab was sufficient to cause dysregulation of GI mucosal immunity. The pattern of biomarkers of GI mucosal dysregulation, including histology, fecal calprotectin, and antibodies to enteric flora, was distinct from that observed for IBD, suggesting that diarrhea and colitis due to CTLA-4 blockade may represent a distinct clinicopathologic entity. Oral budesonide, which is used in the treatment of CD, did not prevent diarrhea or colitis in this study. However, like IBD, GI irAEs do respond to drug withdrawal and systemic steroids and infliximab.

Abbreviations

CD, Crohn’s disease; CI, confidence interval; CTLA-4, cytotoxic T-lymphocyte antigen-4; GI, gastrointestinal; IBD, inflammatory bowel disease; irAE, immune-related adverse event; pANCA, perinuclear-staining anti-neutrophil cytoplasmic antibody; UC, ulcerative colitis

Acknowledgements

This study was sponsored and funded by Bristol-Myers Squibb Company. The sponsor designed the study, and was involved in the collection, analysis, and interpretation of data. We thank Kelly Bennett, PhD, Suresh Alaparthy, PhD, Zenta Tsuchihashi, and Ms. Amy Ronczka, (all Bristol-Myers Squibb Company employees) for their support and contributions in conducting this study. Ping Zhan, PhD (Bristol-Myers Squibb Company) provided assistance with figures. Editorial and writing
assistance, provided by StemScientific, was funded by Bristol-Myers Squibb Company.

Potential competing interests: Hanlin L. Wang has no conflicts to disclose. David Berman, Susan M. Parker, Jonathan Siegel, Scott D. Chasalow, and Susan Galbraith are employees of Bristol-Myers Squibb Company and also disclose ownership of equity in Bristol-Myers Squibb Company. Jeffrey Weber is a consultant for Altor, Inc., and has received funding from Medarex, Bristol-Myers Squibb, and Mannkind Corp. for clinical research. Stephan R. Targan is a member of the Board of Directors of Prometheus, a privately held gastrointestinal diagnostic and therapeutic company and also discloses an equity position.

References


17. Sands BE. From symptom to diagnosis: clinical distinctions among various forms of intestinal inflammation. Gastroenterology 2004; 126: 1518-1532. (PMID: 15168364)


prophylactic budesonide or placebo during a 24-wk induction phase. Secondary objectives included an examination of histologic or endoscopic biomarkers of diarrhea or colitis and their association with grade 2 or higher diarrhea/clinical colitis, an assessment of serologic markers of IBD, measurement of fecal calprotectin levels, and an examination of polymorphisms in immune-related genes.

**Materials and methods**

**Study objectives**

The primary objective of the CA184-007 study was to estimate the rate of grade 2 or higher diarrhea in patients with advanced melanoma receiving open-label ipilimumab and either prophylactic budesonide or placebo during a 24-wk induction phase. Secondary objectives included an examination of histologic or endoscopic biomarkers of diarrhea or colitis and their association with grade 2 or higher diarrhea/clinical colitis, an assessment of serologic markers of IBD, measurement of fecal calprotectin levels, and an examination of polymorphisms in immune-related genes.

**Trial design and drug treatment**

CA184-007 was a randomized, double-blind, placebo-controlled, multi-center phase II trial in treatment-naïve or previously treated patients (n = 115) with unresectable stage III or IV melanoma, enrolled between December 2005 and January 2007 at 11 North American, European, and South American centers (primarily academic institutions) (4).

Patients received 10 mg/kg ipilimumab as a 90-minute intravenous infusion at weeks 1, 4, 7, and 10, for a total of four doses (induction), and were randomized 1:1 to receive concomitant oral budesonide or matched placebo. Blinded oral study medication was self-administered once daily as budesonide 9 mg or placebo until week 12, then tapered until discontinuation at week 16. Patients without progressive disease and able to tolerate treatment were eligible to receive maintenance ipilimumab 10 mg/kg starting at week 24.

The study was conducted in accordance with the ethical principles originating from the current Declaration of Helsinki and consistent with International Conference on Harmonization Good Clinical Practice. Institutional Review Board or Independent Ethics Committee approval of the protocol at each institution was obtained before initiating the trial. All participating patients (or their legally acceptable representatives) provided written informed consent.

**Study patients**

Patients aged 18 years or older with a histologic or cytologic diagnosis of unresectable stage III or IV malignant melanoma (excluding ocular melanoma) and measurable disease were eligible for inclusion in the study. Other inclusion criteria included an Eastern Cooperative Oncology Group performance status of 0 or 1 and a life expectancy of at least 4 months. Patients with a history of autoimmune disease, including IBD, were excluded. Additional key inclusion and exclusion criteria have been previously reported (4).

**Clinical toxicity assessment and management of immune-related adverse events**

The timing and severity of diarrhea and clinical colitis was monitored in all patients throughout the study. Toxicity was graded by the investigator according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0. Patients kept a diary of bowel movements and GI symptoms.

GI irAEs were managed using a guideline (algorithm) developed as part of the ipilimumab clinical program (28). Briefly, diarrhea considered to be low grade (grade 1 or 2), was treated purely symptomatically using loperamide and fluid replacement. If symptoms persisted, treatment was as for low grade toxicity with the addition of oral corticosteroid, especially if colitis was confirmed, and monitoring of the patient was increased. Upon symptom control, steroid taper over 1 month or more was initiated. If grade 3 or 4 diarrhea was identified, intravenous steroids were administered and tapered over at least one month. In cases where symptoms were refractory to intravenous steroids, treatment with ipilimumab was discontinued and treatment of the irAE with infliximab in a
manner similar to treatment of IBD could be initiated. Infliximab was discontinued once symptom relief was evident.

**Lower GI endoscopy and biopsy**

During weeks 1-2, patients were required to undergo flexible sigmoidoscopies or colonoscopies by a local gastroenterologist with visual evaluation for colitis (defined as focal or diffuse erythema, ulceration, abnormal vessel pattern, contact bleeding, or free bleeding in the lumen) and collection of 3-5 colonic biopsy specimens. The site within the colon for endoscopy was not pre-specified. H&E sections of each GI biopsy were evaluated centrally by GI pathologists (GI Pathology, PLLC; Memphis, Tennessee) for focal or diffuse abnormal findings, relative to findings in a normal healthy individual, in the crypts (crypt distortion, mucin depletion, Paneth cell metaplasia; pyloric metaplasia; eosinophilic, neutrophilic, lymphocytic, or granulomatous infiltration), lamina propria (fibrosis, neutrophilic infiltration, excess eosinophils or plasma cells, granuloma), surface lining epithelium (mucosal erosion, ulceration, sloughing, dysplasia, lymphocytic or neutrophilic infiltration) and other (infectious agents, vasculitis, neoplasm).

A second sigmoidoscopy (or colonoscopy, if appropriate) with colonic biopsy was requested in patients experiencing grade 2 or higher diarrhea or colitis and evaluated by a central pathologist with expertise in IBD for histologic evidence of active colitis, which included neutrophilic infiltration of the lamina propria, cryptitis (neutrophilic infiltration of the epithelium), crypt abscess (neutrophils present in lumen of the crypts), mucosal surface erosion, and ulceration. Signs of chronicity, including dense lamina propria lymphoplasmacytic infiltration, basal plasmacytosis, crypt architectural distortion, Paneth cell metaplasia in the left colon, pyloric metaplasia, and granulomas, were also evaluated. Colitis, when detected, was categorized as diffuse if inflammatory changes were contiguous, or focal if the changes were patchy.

**Antibodies to enteric flora**

Well-characterized IBD markers, including pANCA and antibodies to microbial antigens such as ASCA, OmpC, 12 (Pseudomonas), and CBlr1, were measured by an ELISA using serum samples taken at baseline (before infliximab infusion on day 1) and throughout the induction phase. Patients were considered positive for a given antibody if a positive outcome was observed for at least one time point. Generally a positive titer was defined as the mean ± 2 standard deviations of the healthy controls. Cut-off values (EU/ml) for each ELISA are as follows: ASCA IgA, 20; ASCA IgG, 40; I2, 30; OmpC, 23; CBlr1, 30 and ANCA, 35.

**Fecal calprotectin**

Fecal samples for determination of calprotectin levels, a biomarker for IBD, were collected at least 72 hours before clinic visits during the screening, induction, and maintenance phases and if patients experienced grade 2 or higher diarrhea. The assay used for determination of fecal calprotectin (Calprotectin, Eurostátl, S.p.A., Trieste, Italy) has a sensitivity of 95% and a specificity of 93%. According to the manufacturer, a calprotectin level of >30 µg/g is pathological. Median calprotectin concentrations in healthy individuals were 22 µg/g and 27 µg/g for patients 10-59 years and 60 years or older, respectively (29). Calprotectin levels in active Crohn’s disease or ulcerative colitis levels are regularly above 500 µg/g. Typically, there are no significant differences in the level of calprotectin between active UC and active CD (30).

**Genetic polymorphisms**

With patient consent, blood samples were collected for genotyping of SNPs or deletions in 10 immune-related genes: BTNL2, CCR5, CD86, CTLA-4, IFNAR1, IFNAR2, IFNG, IL23R, NOD2, and PTPN22 (18 SNPs and 2 deletions: rs2076530, rs333, rs28897671, rs1129055, rs2681417, rs1863800, rs231775, rs3087243, rs2257167, rs7279064, rs2430561, rs2069705, rs1004819, rs11209026, rs2201841, rs7517847, rs2066844, rs2066845, rs5743293, and rs2476601). A genotype score for at least one polymorphism was available for 94 treated subjects. Two NOD2 polymorphisms, rs5743293 and rs2066845, were monomorphic or nearly so with a minor allele frequency <2%, and thus excluded from further analysis. Blood samples were also collected for medium-resolution genotyping of the HLA-A and -B loci.

**Statistical methodology**

Sample size was not based on statistical power considerations but was determined for purposes of estimation. An interim safety data review had reported a rate of grade 2-3 diarrhea of 27.5% from a previously conducted trial in patients with stage IV renal cancer treated with multiple doses of ipilimumab 3 mg/kg. With a total of 50 patients in the ipilimumab plus placebo group, an anticipated true grade 2-4 diarrhea rate in the 30% to 40% range would result in a maximum width of the estimated exact 95% CI of 28%. With 50 patients in the ipilimumab plus budesonide group, an anticipated true grade 2-4 diarrhea rate of 15% to 25% would result in a maximum estimated exact 95% CI width of 26%.

To ensure consistent assessment of the onset, number, duration, and time to resolution of irAEs (drug-related) and inflammatory (immune-mediated regardless of relationship) events, investigator-reported AEs that were contiguous or occurred between two doses were aggregated into a single event of longer duration.

To assess variability in levels of antibodies to enteric flora within subjects over time, plots of the within-subject standard deviation vs. within-subject mean by worst grade GI irAE were generated. To assess increases in fecal calprotectin over time, a linear mixed model was fitted to fecal calprotectin measurements taken at baseline through the end of week 12. Calprotectin was modeled as a straight-line function of days since first dose. Within-subject correlations were modeled using a spatial exponential structure. Associations between biomarkers and safety measures were analyzed in all treated patients. Genetic associations were analyzed by fitting linear logistic regression models to predict worst-grade GI irAE (grade 2 or higher vs. not) as a function of genotype (for SNPs and deletions) or presence of particular alleles (for HLA polymorphisms). Rates of grade 2 or higher diarrhea or clinical colitis during induction by early signs of histologic and endoscopic colitis were summarized, and corresponding exact two-sided 95% CIs were calculated using the method of Clopper and Pearson (31). The odds ratio for occurrence of grade 2 or higher diarrhea/colitis in the induction phase vs. presence of any early sign of histologic colitis was calculated with exact two-sided CIs based on the method of Thomas (32).