Microbial and Histopathologic Considerations in the Use of Mouse Models of Inflammatory Bowel Diseases

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Abstract: Mouse models provide powerful tools to investigate disease mechanisms and are widely used in inflammatory bowel disease research. However, it is common for reports of mouse model studies to lack potentially important information about the microbial status of the mice and the method used to evaluate disease expression for statistical analysis. For example, it is common practice to state that the mice were housed under specific pathogen-free conditions but provide no further information regarding the presence or absence of organisms such as *Helicobacter* spp. that are known or likely to affect disease expression, thus omitting information potentially important to the expected phenotype of the mice and their responses to experimental manipulation. We therefore encourage authors to use such terms as ''conventional'' and ''specific pathogen-free'' precisely, to state the agents from which the mice are represented to be free, and to provide a brief description of the health monitoring protocol. Descriptions of histopathologic methods used to evaluate colitis in mouse models also often do not include sufficient detail to allow readers to understand and evaluate the methods; in addition, the lesions commonly are shown in photomicrographs that are too small and of too low resolution to be interpreted. Inasmuch as such methods are often the major or only source of data upon which conclusions regarding genotype or experimental treatment effects are based, the method employed should be fully described, and photomicrographs should be of adequate size and resolution to allow independent assessment.

(Inflamm Bowel Dis 2012;18:1558–1565)

Key Words: mouse models, microbiology, histopathology

As readers of *Inflammatory Bowel Diseases* are aware, there is a considerable volume of literature derived from research using mouse models of inflammatory bowel disease (IBD) based on a wide variety of induced mutations, T-cell transfer, selective breeding, and other experimental manipulations, as discussed in recent reviews.[1–4] Our purpose here is to draw attention to certain considerations related to microbial status and histopathologic analysis of disease expression we consider to be important.

MICROBIAL STATUS

Most mouse IBD models are dependent on complex interactions of innate and adaptive immunity with an incompletely understood intestinal microbiota.[1–7] In a landmark article Kuhn et al[8] reported that IL-10 deficient (*Il10*^{tm1Cgn} homozygous) mice "kept under conventional conditions" spontaneously developed enterocolitis, and that IL-10–/– mice transferred into the "defined microbial environment" of a "specific pathogen-free (SPF) facility" expressed less severe disease. No information regarding the microbiological and parasitological status of either the SPF or conventional mice was provided, other than the statement that "mutant mice raised under conventional conditions were free of common intestinal pathogens," without stating what those pathogens were or the test procedures establishing their absence. Similar observations were reported in studies of other mutant mice spontaneously developing colitis, and it soon became, and has since remained, widespread practice in reporting studies using mouse IBD models to state that the animals were housed under SPF conditions but provide no further information about microbial status.

We believe that misconceptions and misunderstanding regarding the meaning and significance of terms such as "conventional" and "SPF" are common. When the use of SPF animals in research became widely accepted in the 1980s, the term "conventional" was used to distinguish SPF animals from those produced and housed in the conventional manner of the time, that is, breeding colonies that were not established from disease-free stock and in which no disease control measures or routine health monitoring were

Received for publication December 16, 2011; Accepted January 4, 2012.

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Partially supported by National Institutes of Health grants 5R24DK064400 ("Mucosal HIV and Immunobiology Center") (to P.D. Smith), 1P01DK071176 ("Innate and Adaptive Immunity in IBD") (to C.O. Elson), and a Crohn's and Colitis Foundation of America grant (to D.C. Bullard).

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DOI 10.1002/ibd.22892

Published online 31 January 2012 in Wiley Online Library (wileyonlinelibrary.com).

implemented. Such animals harbored microorganisms and parasites that interfered with the research in which the animals were used.[9–12] Recognition of such problems was the impetus for development and use of SPF animals, which are free of selected, specific pathogenic and opportunistic microorganisms and parasites considered causes, or potential causes, of adverse effects, whether via morbidity, alteration of biological responses, or otherwise. It is quite inaccurate to equate "SPF conditions" with a "defined microbial environment." Only those agents for which testing is conducted are defined, by their presence or absence according to the testing protocol and results, and such information defines only a few of the large number making up the microbiota.

Conventional animals can be made SPF by a number of methods, including embryo transfer and "Caesarean" (hysterectomy) derivation, but all involve transfer of pups or embryos to mothers that are germfree, gnotobiotic ("known life"—having limited, defined microbiota), or otherwise established to be free of the agents of interest. Breeding populations are established from the derived animals in facilities designed and operated to reduce the risk of contamination ("barrier maintained") and regularly monitored for the agents to be excluded. Such animals acquire a complex microbiota that is somewhat less diverse than that of conventional animals[13] but is otherwise undefined. Furthermore, the composition of the microbiota is influenced not only by diet and environmental factors but also by genotype, sex, familial relationship among breeders, human contact, and even photoperiod.[14–22] Consequently, simply stating that animals were housed under SPF conditions or in a barrier facility conveys only that their microbiota probably differs from that of the conventional stock from which they were derived, but how it differs, and, importantly, whether differences in its composition could influence disease expression in IBD model mice are unknown.

We also encounter the misconception that a standardized "exclusion list" (the "specific pathogens" from which the animals are represented to be free) exists. Certainly, there is broad agreement regarding a handful of the most notorious agents, such as mouse "hepatitis" virus (a group of related mouse coronaviruses), but one can only know which of two or three dozen agents are included in a specific program from the protocol employed by that program. Such information is available on request from all reputable vendors and most research institutions. Vendors' lists typically are quite comprehensive, whereas those of research institutions vary considerably, and can include different "levels" of SPF, that is, different lists of excluded agents, to accommodate different research needs. Thus, it is important to be aware that a given mouse population can be SPF according to the protocols of such a program, but without more specific information that does not necessarily convey whether or not the population harbors agents such as *Helicobacter* spp. that can affect expression of cecocolitis in mouse IBD models.[23–25]

Helicobacter hepaticus and *H. bilis* are well recognized to have important effects on disease expression in mouse IBD models.[4–6,23,24] Other *Helicobacter* spp. that naturally infect mice include *H. ganmani, H. magdeburgensis* sp. nov., *H. mastomyrinus, H. muricola* sp. nov., *H. muridarum, H. pullorum, H. rodentium*, and *H. typhlonius*, and several incompletely characterized isolates that possibly represent additional species.[24–32] *H. trogontum*, originally isolated from rats, infects mice and promotes colitis experimentally.[26,33] These organisms, especially *H. hepaticus* and *H. bilis*, typically promote disease expression in mouse IBD models, but *Helicobacter* spp. can have different effects in mice having different mutations, genetic backgrounds, or microbiota. For example, *H. hepaticus* induced severe disease in C57BL/6 IL-10–/– and C57BL/10 IL-10–/– mice,[35,36] but did not induce colitis in germfree IL-10–/– mice of mixed B6;129 or inbred 129/SvEv background, and germ-free B6;129 IL-10–/– mice inoculated with feces from mice with or without *H. hepaticus* infection rapidly developed cecocolitis of equal severity.[34] In FVB.129P2-*Abcb1a*^{tm1Bor} (*Mdr1a–/–*) mice, disease progression is delayed by *H. hepaticus* infection, but accelerated by *H. bilis* infection.[37]

Although *H. hepaticus* can induce cecocolitis in nude, SCID, and recombinase activating gene-deficient mice,[38–40] it did so in gnotobiotic C.B-17 *Prkdc*^{scid} mice with limited, defined microbiota (Altered Schaedler Flora, ASF) only after CD45RB(high) T-cell transfer.[41] In our hands, gnotobiotic C.B-17 SCID and B6.129S7-*Rag1*^{tm1Mom} mice colonized with ASF and either or both *H. hepaticus* and specific colitis-associated bacteria[42] remain healthy and have no colitis until given T cells, whereupon they rapidly and consistently develop colitis (Fig. 1). Germfree C.B17 SCID mice colonized only with *Helicobacter muridarum*, which, unlike *H. hepaticus*, is not known to cause spontaneous disease,[25] developed colitis after transfer of CD45RB(high) T cells.[43] Other bacteria also can specifically promote disease expression in this model. BALB/c SCID mice colonized with segmented filamentous bacteria (SFB) in combination with a limited microbiota of 12 bacterial species developed colitis after T-cell transfer, whereas mice colonized with either SFB alone or with the limited microbiota without SFB did not.[44]

Mice are often colonized with more than one *Helicobacter* species,[27,28,45–47] and combinations of species can have effects different from those of individual species. Colonization with both *H. hepaticus* and *H. bilis* promotes progression to dysplasia and invasive carcinoma in *Mdr1a–/–* and 129S2/SvPasIco-*Smad3*^{tm1Par} (Smad3 deficient) mice.[48,49] *H. rodentium* alone causes little or no colitis but exacerbates colitis in combination with *H. typhlonius* or

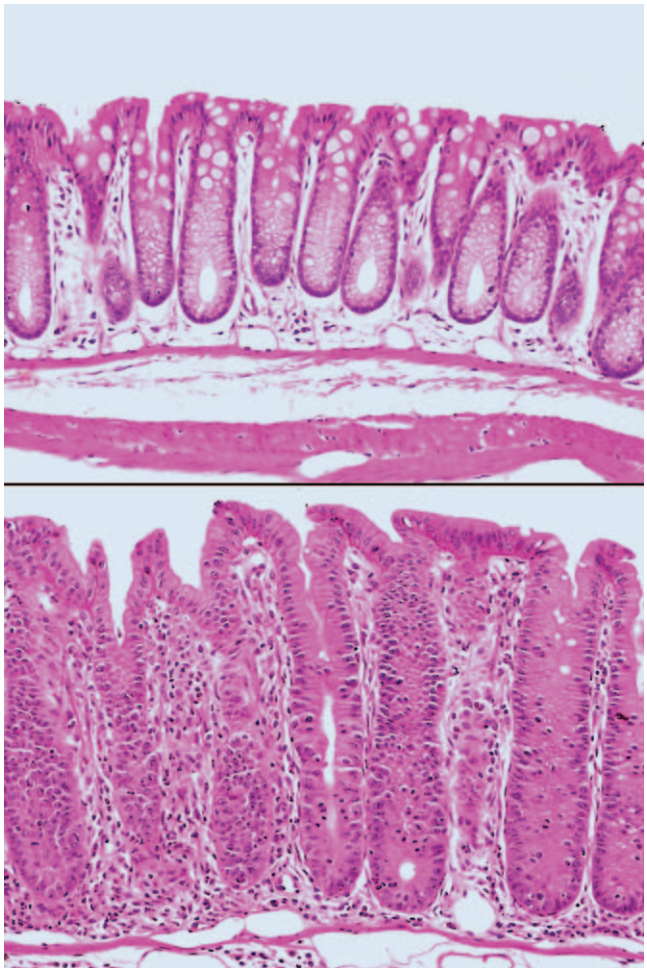

FIGURE 1. Top, normal colon of C57BL/6 *Rag1*–/– mouse colonized with Altered Schaedler Flora and *Helicobacter hepaticus*. Bottom, colitis 3 weeks after transfer of CD45RB(high) T cells into a C57BL/6 *Rag1*–/– mouse colonized with Altered Schaedler Flora and *Helicobacter hepaticus*.

H. hepaticus,[50,51] and, in IL-10–/– mice, accelerates development of colon cancer.[52] Adding to the complexity of potential interactions, infection with mouse norovirus can promote expression of colitis in *Mdr1a*–/– mice colonized with *H. bilis*,[53] and colonization with *Helicobacter* spp. can affect the distribution of other microbiota.[26,54,55] Other organisms have been shown to promote, or, in some cases, inhibit colitis in rodents having various colitis-associated mutations and different combinations of intestinal microbiota, including *Bacteroides distasonis, B. vulgatus, Bifidobacterium animalis, Enterococcus faecium, E. faecalis, Escherichia coli, Klebsiella pneumoniae, Lactobacillus plantarum, Proteus mirabilis*, and *Cryptosporidium parvum*.[56–64] In summary, suffice it to say that the presence or absence of a single bacterial species can dramatically alter colitis expression, the same organism can have different effects in different models, and the effect of a given organism can be dependent on the "context" of the microbiota, that is, the combination of other organisms present.

ANALYSIS OF DISEASE EXPRESSION

In many studies using mouse IBD models, a "semi-quantitative" histopathologic scoring system is used to assess disease expression for statistical analysis. The protocols used vary widely. Unfortunately, some publications do not explain the method in enough detail that an interested reader can understand precisely how the procedure was conducted. To illustrate this point, we recently searched PubMed using the statement "(mice[TI] OR mice[MAJR]) AND (colitis OR "inflammatory bowel diseases"[MH]) AND (scores OR scoring)," resulting in 119 citations. Of these, we selected 34 reports of studies using genetic or T-cell transfer rodent models and tabulated whether the description of the method included the scale of scores; the criteria for assigning each score; how the score or scores for each mouse were derived (whether a single overall assessment was made or a system of component lesions was used, and, if the latter, whether the method of calculation was given); the number and identity of intestinal segments examined; whether the distribution of lesions within or among segments was taken into account; whether the observer had specific qualifications in anatomic pathology; and, if previous publications were cited, whether the cited publications provided adequate additional information.

Of these 34 articles, 32 included a scale of scores, 24 provided at least a minimal description of score criteria, and 17 explained clearly how the score for each mouse was derived. Seventeen articles identified the segments of intestine examined, two specifically included assessment of the distribution of lesions, and 13 stated that evaluations were done by a pathologist. Of 26 articles citing the scoring method of a previous publication, in only two cases did the cited publications provide all of the missing information. In all of the studies, the experimental classifications were concealed from the observer.

Clear communication of the method of assessment also requires anatomic accuracy. Reports of mouse IBD model studies often identify parts of the mouse colon as ascending, transverse, and descending, as do some textbooks on mouse biology. This would be of mere academic interest if there were a clear anatomic correspondence between the large intestines of mice and humans, but that is not the case. In mice, the cecum is comparatively much larger, and is located on the left, with the base caudal.[65] The colon extends rostrally and to the right from the base of the cecum to the region of the pylorus, where it reverses direction by passing around the root of the mesentery to the left and extends caudally to the rectum. Thus, the transverse segment, if it can be said to exist at all, is limited to the very short apex of the reversing curve, and the relative

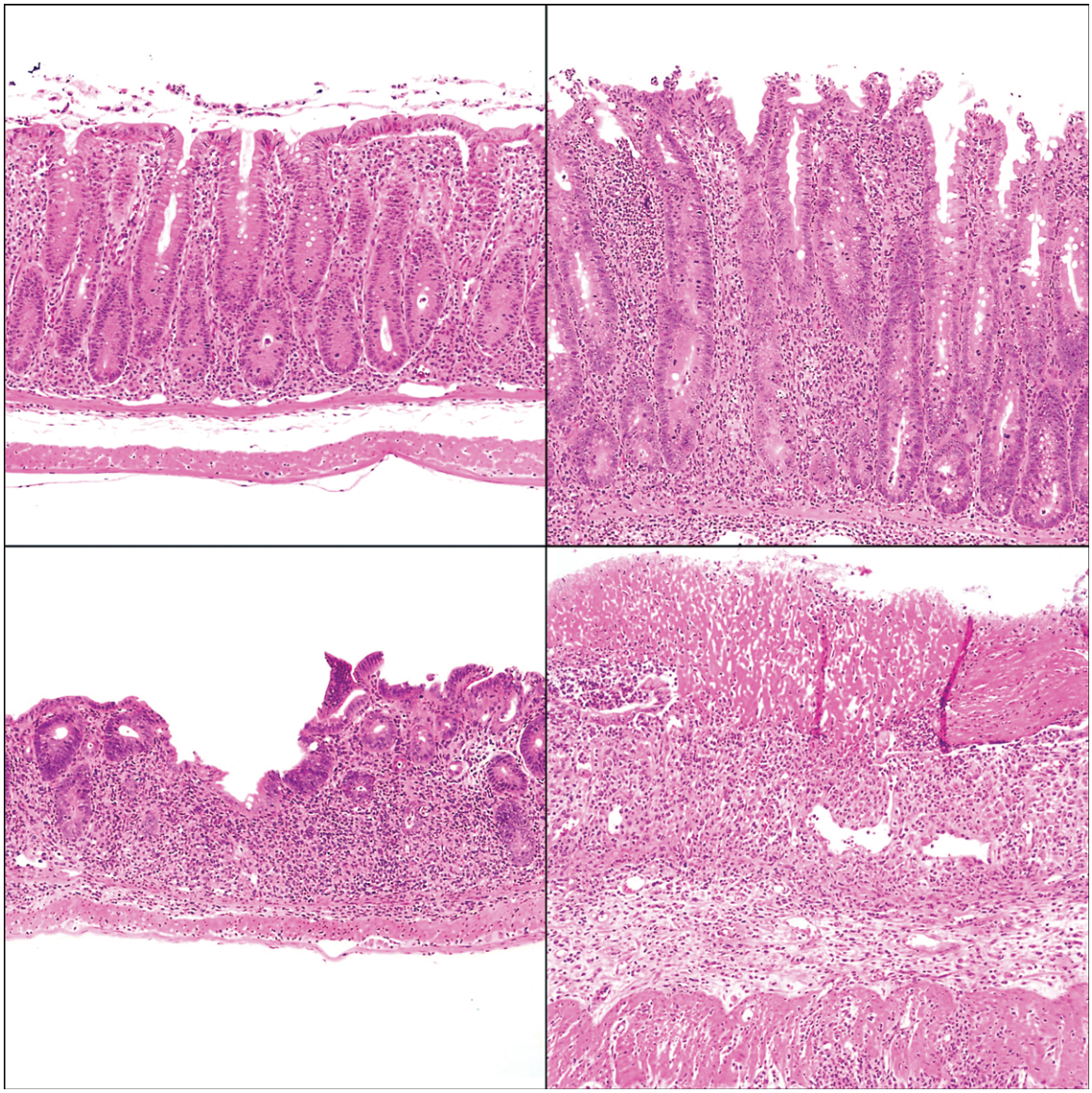

FIGURE 2. Patterns of colitis in mouse IBD models. Top left, mild proliferative colitis. Top right, moderately severe colitis with extensive crypt epithelial proliferation. Bottom left, severe colitis with partial loss of crypt and superficial epithelium. Bottom right, extremely severe colitis with complete epithelial loss and fibrinous surface exudate. Scoring systems should be designed to appropriately evaluate colitis in which epithelial changes cannot be scored because of epithelial destruction.

proportions of the ''ascending'' (rostrad) and ''descending'' (caudad) segments in mice are considerably different from those of the ascending and descending colon in humans. We prefer to avoid imprecise anthropomorphic terminology and simply divide the colon into proximal, middle, and distal thirds, which is unambiguous and corresponds reasonably well to normal mucosal histology.

Published scoring methods vary greatly in criteria and complexity. We consider the most important consideration to be the accuracy with which the method reflects the disease manifestations of interest. A difficulty we have encountered with scoring protocols is that, unless carefully designed, their ability to generate reliable overall severity assessments can be dependent on whether the experimental comparison involves a fundamental change in the character of the response. In the case of genetic and T-cell transfer mouse IBD models, mucosal inflammatory cell accumulation and crypt hyperplasia are generally considered to be primary features of cecocolitis, such that increasingly heavy inflammatory cell accumulation and increasingly pronounced hyperplasia are logically taken to be major indicators of increasing overall severity. However, as the severity of epithelial injury increases, epithelial degeneration and loss begin to reduce the apparent contribution of

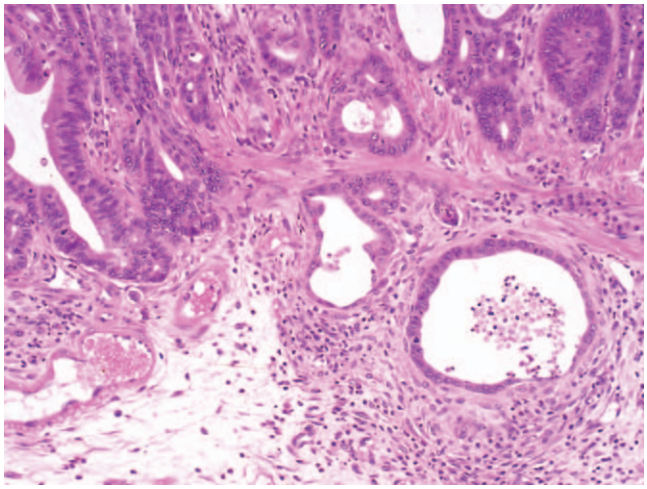

FIGURE 3. Colitis induced by *Helicobacter hepaticus* in an immunodeficient mouse, with proliferating epithelium penetrating the muscularis mucosa and extending into the submucosa, a nonneoplastic change occurring in chronic severe colitis induced by this organism.

proliferation, and, if severe enough, lead to progressive crypt loss and eventually complete epithelial loss and lamina propria "collapse" (Fig. 2). If an experimental manipulation alters expression of cecocolitis such that it becomes primarily necrotizing rather than proliferative, a scoring protocol that sums scores for, say, goblet cell loss, epithelial hyperplasia, crypt exudate ("abscesses"), crypt loss, and lamina propria inflammatory cell accumulation will yield representative total scores only insofar as all of the structures in question are present. If the epithelium is lost, goblet cell loss, crypt hyperplasia, and crypt exudate cannot be assessed and cannot contribute to the total score, an effect that will be only partially offset by increasing crypt loss scores. Thus, a total score so derived may not adequately reflect the difference between a hyperplastic mucosa and a more severely affected one with extensive epithelial loss. Such potential problems can be accommodated by weighting component scores or other means. We also consider it advisable to include specific assessment of lesion distribution, as intestinal lesions in mouse IBD models can be discontinuous, especially in early or less severe disease, and can differ between cecum and colon and among segments of colon. In addition, we have seen instances in which a genetic or experimental manipulation significantly affected disease expression in some segments but not others. A final consideration is the progression of colitis with time. We have encountered cases in which an experimental manipulation did not significantly alter the severity of colitis present at the end of an experiment, but did significantly affect colitis development, that is, severity at earlier timepoints. Such differences could point to subtle but potentially important biological effects; therefore, we suggest that a time course study of disease progression may be appropriate whenever a new combination of mouse model and experimental manipulation is investigated, particularly if an initial comparison at a typical duration of several weeks is negative.

In any case, the objective should be a distribution of scores that reflects a pathologist's assessment, that is, lesions judged to be mild, moderate, or severe should be represented by progressively higher scores separated by appropriate intervals. Meeting this objective requires accurate interpretation of each of the lesion characteristics to be evaluated. For example, transmural inflammation, an inflammatory process that directly invades and penetrates the tunica muscularis, damages smooth muscle, and extends to the serosa, is characteristic of severe or aggressive disease and is reasonable to include as an important component. However, mucosal inflammation in mouse colon and cecum commonly is accompanied by phlebitis, lymphangitis, and perivascular inflammatory cell accumulation affecting vessels traversing the tunica muscularis, which can be associated with serositis, especially at mesenteric attachments. Although this could be interpreted as transmural inflammation in a sense, it is a different process of quite different significance. Similarly, extensive epithelial loss resulting in lamina propria collapse is different from ulceration, in which loss of mucosal tissue extends into the submucosa, although the lesions can have a similar appearance at low magnification if the ulceration does not extend into the muscularis. Another example is mouse IBD model studies in which development of cancer is of interest. Here it is important to be aware that it is characteristic of mouse colonic mucosa that epithelial hyperplasia is accompanied by a tendency for proliferating crypt epithelium to penetrate the muscularis mucosa where inflammation is severe or prolonged, such as that often associated with *Helicobacter* spp.[66] (Fig. 3). The proliferating and invading epithelium can appear quite dysplastic, making such lesions difficult to distinguish from early invasive carcinoma.[66,67] For studies in which cancer development is of interest, reference to published criteria for distinguishing such lesions from carcinoma[67] may be helpful. In addition to the problem that the method of histopathologic assessment may not be clearly presented, it may not be possible for the reader to independently assess the reported disease manifestations because they are not adequately illustrated. In our view, by far the most common cause of this is the practice of "postage stamp pathology"[68]—publication of photomicrographs so small and of such low resolution they cannot be interpreted.

CONCLUSIONS

A commonly cited standard for research articles is that enough information be provided that a competent

investigator could repeat the work.[69] In our view, the information regarding the topics discussed here provided in reports of mouse IBD model studies often falls short of this standard. We encourage use of "conventional," "SPF," and other such terms in a manner that reflects their precise meaning. Stating that animals were housed under SPF conditions without providing health monitoring results may well omit information important to the expected phenotype of the mice and their responses to experimental manipulation; therefore, the agents from which the animals are represented to be free should be stated, and a brief description of the testing protocol provided or an informative reference cited. Of particular importance, presence or absence of organisms such as *Helicobacter* spp. that are known or likely to affect disease expression should be documented to provide some basis for assessing reproducibility among studies with a given model, particularly in cases in which specific features, such as cancer induction, are of interest. Basic characterization of *Helicobacter* spp. colonization status is readily accomplished by commercial polymerase chain reaction (PCR) testing for *H. hepaticus, H. bilis*, and generic *Helicobacter* spp. Specific methods for other species have been described[27,28,70–73] and are well within the capabilities of most biomedical research institutions.

Inasmuch as histopathologic assessment is often a major, if not the only, method generating the data upon which conclusions regarding genotype or experimental treatment effects are based, we think a concise but complete description of the method employed should be required in all reports of studies of mouse IBD models. At a minimum, if a previous publication is cited it should provide the necessary information without requiring the reader to follow a chain of citations back to the original publication of the method, which itself may not provide that information. We agree with others[74] that a more complex system of histopathologic assessment can be advantageous. We use a multiple component system designed to accommodate different lesion patterns, allow separate analysis of component lesions, and generate data more likely to be suitable for parametric statistical analysis with its attendant power advantage and ease of multiple comparisons. However, in our experience, design and use of such systems are not simple tasks—we have made significant changes over the years to improve flexibility and accommodate different models—and require formal anatomic pathology skills and experience in "analytical pathology" of mouse IBD models. We are aware that such expertise is not always available, but resources exist that may help identify potential collaborators, such as the Center for Genomic Pathology (http://ctrgenpath.net/) and the Johns Hopkins University Phenotyping Core (http://www.hopkinsmedicine.org/mcp/PHENOCORE/). Finally, the value of any analysis is compromised by publication of poor photomicrographs. In our view, journals should require that photomicrographs be of adequate size and resolution to allow independent assessment by the reader, if necessary providing such illustrations as online supplemental material.

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