Using the “Smurf Transition” to Study Leaky Gut in a Fruit Fly Model

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Teacher Guide

Science Background

Our bodies are an ecosystem unto themselves. Living in and on you are trillions of tiny microbes and, much as in the natural ecosystems around us, the composition of our "microbiome" can shape and be shaped by environmental disruptions. Antibiotics may act like pesticides, wiping out the "good" species along with the "bad" ones and changing the structure and function of the ecosystem.[1] The same is true for hygienic products marketed for their "germ killing" properties. On the other hand, being exposed to the microbiota of farm animals appears to be beneficial – the earlier in life, the better.[2] In fact, comparisons of health outcomes in children delivered by caesarian to children experiencing a conventional vaginal delivery are revealing that the earliest opportunity most of us probably have to acquire a healthy microbiome is when we pass through our mother's birth canal.[3]

The fact that we are the habitat for such a multitude of microorganisms and that this is a good thing surprises many people who are accustomed to thinking of microorganisms as disease agents and antimicrobial cleaners as part of a vital defensive arsenal. Nonetheless, there are sometimes rogue species among the hordes, and we learn about their effects in breaking news stories and food product recalls when people suffer from pathogen outbreaks. Having a diverse microbiome may actually be of some help in protecting us from some of these threats because, in general, diverse ecosystems tend to be more resistant and resilient to invasion by new species. But there's an additional level of protection that our microbiome helps to produce. It turns out that our epithelium, the outermost layer of cells that stands between the outside world and our inner spaces, is constantly monitoring our microbiome and culling it with help from our immune system. The sensors these cells use to monitor our guests are downregulated when the microbiome is depopulated or missing and, consequently, the ability of our epithelium to protect us is diminished.[4] Maintaining diverse, healthy microbiota helps keep our epithelial cells and the immune cells that patrol among them vigilant.

This is especially true in the mucosal epithelium which lines our intestine. These cells secrete a layer of mucus that keeps most microbes at a safe distance, but this mucus also contains food for the beneficial microbes to sustain a limited population of them when our diets aren't providing sufficient quantities of their substrates.[5] Some of these cells also constantly produce antimicrobial products to keep microbe populations in check.[6] Since this epithelium evolved to absorb nutrients, it is especially prone to invasion, and the rapid turnover of cells may minimize their risk of becoming pathogen nurseries.[7] The cells here also have morphologically distinct sides with a unique membrane composition on the side exposed to the lumen of the intestine compared to the lymphatic side (opposite the lumen). The tight junctions that prevent leakage of lumen contents into the lymph help to maintain this membrane polarity and, when these tight junctions are disrupted (as may occur in inflammatory bowel diseases or IBDs), the ability to detect material that escapes the lumen and enters the lymph enables these epithelial cells to signal the immune system and other epithelial cells to initiate protective and reparative responses. Some of the researchers who study inflammatory bowel diseases are looking to the microbiome and our dynamic interactions with it to find new modes of treatment. They use

model organisms to test their hypotheses about what goes wrong in inflamed or “leaky” guts and to discover ways that these conditions can be avoided or treated with fewer complications.

The workhorse in this research is the mouse and many variants that mimic human genetic vulnerabilities in IBDs are now available; some even express human proteins so researchers can better understand why certain heritable gene variants make patients more susceptible to IBD. Not surprisingly, the malfunctioning proteins are often found to play a role in the maintenance of the tight junctions, production of epithelial secretions (mucus, the complex carbohydrates that support our friends when our diet cannot, and antimicrobial peptides). They may also be the signals or receptors that our epithelial and immune cells use to monitor and regulate our microbiome. These weak links become therapeutic targets. Unfortunately, experimenting with mice is not possible in most high schools so this unit plan was written to utilize fruit flies as a model organism. Fruit flies harbor a diverse assemblage of microbes (albeit less diverse than vertebrates) in a gut with a single-layer of epithelial cells that is continually renewed and that interacts dynamically with the microbiome using some of the same signaling complexes our cells use.[8] The use of fruit flies as a model organism for IBD research is relatively new. For the high school student, the novelty of this model system means more questions remain open to investigation!

To my knowledge, the “smurf transition” I’m introducing to students in this curriculum is the first time it has been used specifically to model inflammatory bowel disorders. It has been used as an early indicator of senescence (old age) because the gut becomes inflamed in aging fruit flies and begins to disintegrate hours or days before flies die, giving researchers opportunities to study a variety of events that precede death - some of which may occur in humans as well.[9] Since it gives such a clear indication of the presence of a leaky gut in fruit flies and uses a simple, non-toxic compound (the ingredient in blue food coloring), it seemed like the perfect tool for students and their teachers to use for studying gut inflammation in a high school lab!

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3. Neu, J. and Rushing, J. 2011. Cesarean versus Vaginal Delivery: Long term infant outcomes and the Hygiene Hypothesis. Clinics in perinatology.38(2):321-331.
4. Round, J. L., and S. K. Mazmanian. 2009. The Gut Microbiome Shapes Intestinal Immune Responses during Health and Disease. Nature reviews. Immunology 9(5): 313–323.
5. Pickard, J.M. and A. V. Chervonsky. 2015. Intestinal Fucose as a Mediator of Host–Microbe Symbiosis. Journal of Immunology 194(12): 5588-5593
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7. Chichlowski, M., and L.P. Hale, L.P. 2008. Bacterial-mucosal interactions in inflammatory bowel disease: an alliance gone bad. American Journal of Physiology. Gastrointestinal and Liver Physiology. 295:G1139–G1149
8. Chandler, J.A., J.M. Lang, S. Bhatnagar, J.A. Eisen and A. Kopp. 2011. Bacterial Communities of Diverse *Drosophila* Species: Ecological Context of a Host–Microbe Model System. PLoS Genetics 7(9):e1002272.
9. Dambrose, E., L. Monnier, L. Ruisheng, H. Aguilaniu, J.-S. Joly, H. Tricoire and M. Rera. 2016. Two phases of aging separated by the Smurf transition as a public path to death. Scientific Reports 6: 23523.

Student Outcomes

- Students will develop a laboratory model of gut inflammation (“leaky gut”) using a fruit fly.
- Students will develop a conceptual model of gut inflammatory reactions in terms of the gut epithelial structure and dysregulation in cell signaling and response.
- Students will model the dynamic interactions between gut epithelial cells, lymphatic cells and the microbial ecosystem in the gut lumen.
- Students will learn how to read a scientific paper.
- Students will learn about the tools biologists use to study the biological activities of a cell and how they are affected by events in their environment.
- Students will design and defend a research proposal involving the use of modern molecular tools to study gut mucosal immunology in a fruit fly.

Course Placement

This unit plan was designed to be used in a unit on cell interactions/cell signaling in AP Biology but parts of it could be used in a lower level course or for enrichment in an advanced course.

AP Biology Curriculum Standards

Essential knowledge 2.C.1: Organisms use feedback mechanisms to maintain their internal environments and respond to external environmental changes. (b&c dysregulated inflammatory responses)
Essential knowledge 2.D.1: All biological systems from cells and organisms to populations, communities and ecosystems are affected by complex biotic and abiotic interactions involving exchange of matter and free energy. (b&c)
Essential knowledge 2.D.3: Biological systems are affected by disruptions to their dynamic homeostasis. (a&b)
Essential knowledge 2.D.4: Plants and animals have a variety of chemical defenses against infections that affect dynamic homeostasis. (a&b)
Essential knowledge 2.E.3: Timing and coordination of behavior are regulated by various mechanisms and are important in natural selection. (b4)
Essential knowledge 3.B.2: A variety of intercellular and intracellular signal transmissions mediate gene expression. (a&b)
Essential knowledge 3.D.1: Cell communication processes share common features that reflect a shared evolutionary history. (a&d)
Essential knowledge 3.D.2: Cells communicate with each other through direct contact with other cells or from a distance via chemical signaling. (a-d)
Essential knowledge 3.D.3: Signal transduction pathways link signal reception with cellular response. (a&b)
Essential knowledge 3.D.4: Changes in signal transduction pathways can alter cellular response.
Essential knowledge 4.A.3: Interactions between external stimuli and regulated gene expression result in specialization of cells, tissues, and organs. (c)
Essential knowledge 4.A.4: Organisms exhibit complex properties due to interactions between their constituent parts. (interactions between integumentary and immune systems)

Essential knowledge 4.A.5: Communities are composed of populations of organisms that interact in complex ways. (a&b)
Essential knowledge 4.A.6: Interactions among living systems and with their environment result in the movement of matter and energy. (c, e2)
Essential knowledge 4.B.2: Cooperative interactions within organisms promote efficiency in the use of energy and matter.
Essential knowledge 4.B.3: Interactions between and within populations influence patterns of species distribution and abundance. (a-c applied to interspecific interactions among the microbiome)
Essential knowledge 4.C.1: Variation in molecular units provides cells with a wider range of functions. (applies to differences in TLRs,NODs, xenosensors)
Essential knowledge 4.C.4: The diversity of species within an ecosystem may influence the stability of the ecosystem. (a&b)

AP Biology Science Practices
Science practice 1: The student can use representations and models to communicate scientific phenomena and solve scientific problems.
Science Practice 3: The student can engage in scientific questioning to extend thinking or to guide investigations within the context of the AP course.
Science Practice 4: The student can plan and implement data collection strategies appropriate to a particular scientific question.
Science Practice 5: The student can perform data analysis and evaluation of evidence.
Science Practice 6: The student can work with scientific explanations and theories.
Science Practice 7: The student is able to connect and relate knowledge across various scales, concepts and representations in and across domains.

Time Requirements
The activities included in this unit will require approximately 7 days (90 minute periods) but can be broken up and used in discrete pieces and even across different units.

Advance Preparation
Two fruit fly cultures should be prepared at least one week in advance. One should include fruit flies that are grown on standard medium and transferred one day before the first session to a culture vial with medium containing 2.5% FD&C Blue No. 1 (also called Brilliant Blue FCF 1, this is the most common blue dye used in coloring foods). The second culture should be treated exactly the same with the exception that the starting medium will be prepared with 2% DSS (Dextran Sulfate Sodium Salt) solution.
Additional stock fruit fly cultures should be prepared for each student's summative investigation a week before the students begin these experiments.

The following papers should be downloaded and a copy printed for each student – in color if possible – prior to the beginning of the unit:

Fasano, A. 2009. Surprises from celiac disease. Sci Am. 301:54-61.

Velasquez-Manoff, M. 2015. Gut microbiome: the peacekeepers. Nature. 518:S3-S11.

Abreu, M.T. 2010. Toll-like receptor signaling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol. 10:131-44.

Philpott, D.J., M.T. Sorbara, S.J. Robertson, K. Croitoru and S.E. Girardin. 2014. NOD proteins: regulators of inflammation in health and disease. Nature Reviews. 14: 9-23.

Hong, C. and P. Tontonoz. 2008. Coordination of inflammation and metabolism by PPAR and LXR nuclear receptors. Current Opinions in Genetics and Development. 18:461-467.

Materials and Equipment

The materials I used in developing this unit plan were either standard resources the teacher will probably already have (e.g. dissecting microscopes) or are inexpensive. Many pet owners raise their own fruit flies to feed their pets so it's relatively easy to find simple instructions for inexpensive fruit fly feeding media, and your local pet store will probably be able to supply flightless fruit flies. The dye used to detect a leaking gut is actually food coloring and the concentration of that obtained in a grocery store will work just fine. Also, DSS is the standard chemical used to induce inflammation in the guts of laboratory animals, but I recommend dish soap as it will be much less expensive and will work fine for your purposes. I mixed ten drops of Palmolive™ dish soap with the contents of a 0.25oz bottle of blue food color to induce gut disruption. Finally, many teachers prefer to use CO2 from antacid tablets as a safer way to anaesthetize flies.

Price list for unit materials:

Item	Supplier	Price
Fruit fly culture medium (1L)	Carolina Biological Supply	8.95
Fruit fly vial set (72 vials)	Carolina Biological Supply	88.50
Wild type Drosophila (25-30)	Carolina Biological Supply	7.95
Flynap Anesthetic kit	Carolina Biological Supply	13.75
Dextran Sulfate Sodium (DSS)	Sigma-Aldrich	80.80
Brilliant Blue FCF (FD&C Blue #1) (100mg)	Sigma-Aldrich	53.50

Self-Stick Easel Pads (4 - 30pg tablets)	Crystal Rock Office Supplies	76.41
Crayola Poster Markers (price per 8pk)	Crystal Rock Office Supplies	5.54

Student Prior Knowledge and Misconceptions

Prior Knowledge: Students should know something about the structure and function of the digestive system, and the roles of the immune system in protecting us from pathogens and in inflammatory and allergic reactions. There is an excellent set of *Crash Course* videos on Youtube that students can use to review the immune system; a viewing guide has been included in the appendix. Students should also have a basic understanding of cell structure and function – especially receptor-ligand interactions and signal transduction as they relate to activation of gene expression.

Misconceptions: Interestingly, most if not all of my students already knew something about the microbiome but they think of it in terms of "good" microbes and "bad" and this dichotomy is too simplistic. This provided an opportunity to talk about the gut microbiome as an ecosystem with competition, mutualisms and commensalisms. We talked about how a symbiotic relationship can shift, and how too high density of even the "good" bacteria may actually be a "bad" thing. It's important to note that even species we refer to as commensals can be opportunistic, and can go rogue as sometimes occurs with *Staphylococcus* species.
Additionally, most students probably won't know that irritable bowel syndrome (IBS) is a different condition than IBD. The teacher may want to discuss the differences and similarities but should at least make note of this fact to help students avoid distraction from irrelevant information while doing online research. Finally, mid-way through the year of developing this curriculum, new estimates emerged showing that the conventional 10:1 bacterial to human cell count was inaccurate; a 1:1 ratio is probably more realistic (and still quite impressive!). Students who knew about the microbiome mostly were not aware of this change, and for at least the next few years, many of the resources they will encounter online will be using the old estimate, which is off by an order of magnitude!

Daily Unit Plans

Prior to Day 1

1. At the end of prior unit, hand out *Crash Course* questions and assign students to watch videos on the lymphatic system and the immune system and answer questions as homework due at the beginning of class on day 1.

Day 1

1. Ask students if they have any questions about the *Crash Course* videos, answer them, then collect their homework
2. Assign students into working pairs or small groups and give them anesthetized flies from intact and leaky gut treatments to view under the microscope.
3. Show informational video about Inflammatory Bowel Disorder (IBD) and have students create models (posters with markers) to account for the differences in appearance between the two fruit flies.
4. Distribute "Surprises from Celiac Disease" article directing students to study the image on pg. 38 and "Gut Microbiome: the Peacekeepers" in which students will study the image on page S9, "Your Microbes at Work: Fermenters in your gut keep you healthy." Engage class in discussion about what's going on in these images and how it may relate to what they observed under the microscope and in the video.
5. Assign students to read both articles as homework and to consider how they would incorporate this information into their models. (You may choose to use the quiz in the appendix as a "Do Now" at the beginning of the next class period.)

Day 2

1. Students begin class by viewing "Immunology of the Gut Mucosa" video.
2. Starting with a new poster board, students create a model of the human digestive system that incorporates what they have learned. By playing, stopping, and rewinding the videos and referencing the diagrams they have, students make their models reflect as much as they now know about gut mucosal immunology.
3. Students do a "gallery walk" to view the work of other groups and return to make any necessary changes to their own posters. As a class, students discuss their emerging understanding of the gut epithelium and the roles of different kinds of immune cells in regulating inflammatory responses.
4. Students watch a video on TLR receptors in class: https://www.youtube.com/watch?v=GXECgTLGLtI)
5. As homework, students are given the TLR paper, the reading guide and the glossary and assigned a portion on which to become the class "expert."

Days 3 & 4

1. Teacher provides a rough sketch of figure 1 from TLR paper without any labels and students take turns presenting on their area of expertise adding cells and labels to the class diagram as they share the information on which they specialize.

2. When finished with discussion of the paper, students should incorporate new information into their models.
3. Students watch video on NOD-like receptors in class: (https://www.youtube.com/watch?v=HSk90Yx4ulE)
4. Students take home NOD-like receptor and Nuclear Receptor papers and do their best to determine what they're about from looking at and reading the captions for the images. The Butyrate in the "Your Microbes at Work" image from paper given on Day 1 is an example of a nuclear receptor ligand.

Day 5

1. Class begins with discussion of the role of NOD and NRs in immunity.
2. Students conduct internet research to identify factors which may either increase or decrease gut inflammatory response and propose a new experiment to test effects of their factor on speed of Smurf Transition.

Day 6

1. Teacher introduces cell biologist's toolkit.
2. Students begin preparing three minute talks on how they might be able to use these tools to study the effects of their treatments.

Day 7

1. As summative assessment, students present their three minute talks, field questions, and respond to feedback.

Student Section

Rationale

You may already be somewhat familiar with the important job your immune system does in helping you avoid getting sick because you had to show proof of vaccination in order to enroll in school, and you likely get vaccinated every year against the seasonal "bugs." But did you know that your gut is the most important site of defense in your whole body? To understand why this is, consider the fact that the thin layer of cells lining your gut has to provide passage for the molecules you absorb from your food and therefore *must be* more permeable than other epithelial barriers. This greater permeability to food molecules presents a potential passageway for pathogens to enter the body and it is for this reason that natural selection produced such a formidable defensive arsenal along the length of our intestines.

Unfortunately, many people around the globe suffer from excessive activation of these defenses in the presence of low-level threats or even in the complete absence of threats, and they may experience the unpleasant effects of their body's response in the form of Inflammatory Bowel Disease (IBD). Consequently, many researchers are engaged in the work of trying to work out the cellular details of these inflammatory responses so that one day those who suffer from them may be able to enjoy relief from treatments aimed at better controlling the inflammatory pathways.

Since most experiments on humans are considered unethical, researchers who want to better understand these and other disorders impacting human health use other organisms as "models." The mouse is one of the most commonly used models because it's a mammal, and its responses to experimental manipulation are more likely to resemble a human's responses to the same treatments since we share a recent ancestor. However, working with mice at the high school level is almost impossible, so you will use a more distant relative which doesn't currently enjoy the protections that mice do. Your model for studying gut immunology will be the lowly fruit fly, *Drosophila* sp.

Researchers trying to understand what happens in our bodies at the end of life have been using fruit flies as models for many years. While searching for ways to study the cellular events that precede death, they discovered that the gut becomes inflamed and breaks down hours before the fly actually dies and before the fly shows an obvious decline in activity or vigor. To help them identify which flies were undergoing this transition, they found that food dyes could be added to a fly's food without harming the flies, and its leakage into the abdominal cavity could be easily observed under a dissecting microscope. They began using the FD&C blue dye used in coloring food as their marker and dubbed the transition from an intact to a leaky gut the "smurf transition" assay. You'll be using this assay to determine which of your treatments trigger gut inflammation or "leaky gut."

Before you begin this unit, you will watch some videos introducing the lymphatic system and the immune system, and you will answer questions as you watch. This is meant to give you a quick introduction to the function and complexity of the immune system. You will then use a

microscope to look at two fruit flies that have been feeding on a growth medium to which blue food coloring was added. One was also fed in a medium to which a detergent was added. You should be able to see a difference between them. One will have the food coloring localized in its digestive tract and the other will have blue diffused throughout its abdomen.
Through a series of videos, images, and papers that your teacher will share, you and your classmates will develop a working model of inflammatory bowel disorder that can explain these conditions at the level of individual cells. Your models will reflect the array of different possible environmental triggers for inflammation, the receptors and pathways activated by them, and the resulting change in phenotype at the tissue level. After developing this model, you will have a chance to set up your own experiment using fresh fruit flies and propose a more advanced analysis using modern molecular tools to reveal just exactly what may be going on at the level of gene activation within distinct cell populations.

Day 1: Building a Model of Leaky Gut in a Fruit Fly

Smurf Flies! Pair up and obtain each of the two fruit flies that your teacher has prepared for you (one fed on a standard medium with food color added and one fed on a medium with food color and detergent). Quickly inspect both fruit flies using a stereomicroscope (or magnifying glass if stereomicroscopes are not available). Write down your observations. What do you see? (Don't worry about trying to explain why. That's where we're going next!)

What's a "leaky gut?" Now your teacher will ask you to view a brief informational video about Inflammatory Bowel Disease or IBD (https://www.youtube.com/watch?v=Keqzt83KMVA) and you will be asked to work in a group to create a model that connects the effects of IBD to what you observed in the fruit flies. What might the soap do to the fruit fly's gut lining? Do people with IBD consume things that act like soap in their gut but doesn't affect other people? Might there be things in the environment that can cause IBD symptoms in us as well if we consumed them?

What causes a gut to leak? Look at the image on page 38 of the 2009 Scientific American article "Surprises from Celiac Disease" and the image on page S9 of the 2015 Nature article "Gut Microbiome: The Peacekeepers." Discuss with the rest of the class what these images seem to show and what they may contribute to the development of your model of IBD. What happens to the connections between the cells? What do you think the mucus layer does? What are the immune cells are doing?

For homework: Read both of these articles and write some notes for yourself as you read if you see some details that might help you to improve your model of IBD and the role of the gut mucosal epithelium.
Your teacher may either have you watch "Immunology of the Gut Mucosa" (https://www.youtube.com/watch?v=gnZEge78_78) as a class or in your individual groups.

Day 2: The Role of Immune Cell Signaling Mechanisms in IBD

A more detailed look at the defenses of the gut epithelium: Watch the video "Immunology of the Gut Mucosa" (https://www.youtube.com/watch?v=gnZEge78_78) and add details about the dynamic role of the gut mucosal immune system to your model of the gut epithelium. As your previous paper may be getting pretty crowded and messy, you may ask your teacher for a fresh piece of poster paper if necessary.

Walk around! At this point, your group has been working in isolation from other groups to come up with the most comprehensive and up-to-date model of the gut epithelium and your ideas are limited to what the members of your group saw and remembered from the videos, pictures and articles they saw. But are there things you missed? Might other students have seen things or thought for things that your group members didn't? This is a good time to find out! Get up, take your notebook, and walk around to look at the work that has been done by other groups. Write down anything you see that your group didn't include but probably should have. When you get back to your own group's poster, add it in!

Talk about it! Scientists develop their understanding of the natural phenomena they study in much the same way you are – by trying to come up with an explanation for what they see, gathering more information and improving their understanding. But an important part of this process is sharing their discoveries and insights with other scientists and studying the work of other scientists to find out what their colleagues have learned in their own investigations. This exchange often takes place at professional meetings where they can watch each other give presentations and talk to each other while looking at posters that summarize their results. You'll try to emulate this now by discussing your models as a whole class.

Wait! There's more! So far you should have a pretty good idea of the way different cells are arranged within and adjacent to the gut epithelium as well as what the different cell types here do. But you should now be wondering about some of the specific mechanisms involved. For instance, if one immune cell sends a signal and the cell receiving it undergoes a phenotypic change or produces a product of its own in response, how is this happening? Your teacher will now share a video with you about the Toll-Like Receptors or TLRs (https://www.youtube.com/watch?v=GXECgTLGLtI) and for homework you will be assigned to become very familiar with *one portion* of a scientific paper written to review what was known about the function of TLRs at the time the paper was written. To help you, the teacher will provide a reading guide and a glossary for technical terms and acronyms you're unlikely to know. Do the best you can because you'll have to teach your classmates what you know the next time you meet!

We're smarter together: So far you've been adding to your understanding of the gut epithelium somewhat like a scientist might do. The model you're holding in your head has become increasingly sophisticated as you've added details to your understanding. The paper you are being given at the end of this class was full of details; too full, in fact, for you to really get much out of it on your own. But, as we've seen, scientists don't really work alone. Science

is a collaborative process and, when scientists collaborate, they can achieve far more than they ever could have done working in isolation. Because your teacher assigned you to read just one part of the paper and you will become the expert on a certain piece of the TLR response puzzle, your task in the next period will be to show other class members what you have learned and they will do the same.

Days 3&4: Reading a Scientific Paper

Time to put on your teacher-scientist hat! Since visualization helps us all understand things better, you are asked to add your knowledge to a classroom model of the gut mucosal immune system. Take your time. This may take a couple class periods depending on how much you and your classmates have to say! When the class has finished fleshing out the details of Toll-Like Receptor signaling, each group should meet for a few minutes to discuss their models and try to reach a consensus on how this new knowledge may be incorporated into these models. For the next class, students will discuss two other classes of receptors that may play a role in the cellular response to pathogen-derived factors. One of these is a class of intracellular receptors called NOD-like receptors. You'll watch the video about them in class before you leave (https://www.youtube.com/watch?v=HSk90Yx4ulE) and skim through the scientific review paper your teacher gives you about them to get a handle on how they compare to TLRs. This paper is like the one you just read about TLRs but see if you can get the "gist" by flipping through the paper and studying the diagrams. Do the same with the paper on nuclear receptors. These are yet another class of receptor that may enable your cells to recognize and respond to bacteria that are nearby. They're the receptors used by the immune cells to detect the short chain fatty acid metabolites (e.g. butyrate) shown in the figure on page S9 of the "Gut Microbiome" paper you already read.

Day 5: On the Frontiers of Science!

Where do we go from here? In just a few days' time, you connected the escape of food coloring from a fruit fly's gut lumen into its body cavity through breaches in the wall of the gut to an explanation of what happens in the gut of IBD sufferers. You progressed from a pretty rudimentary description of gut epithelial structure to a pretty complex and dynamic view of how the gut barrier is maintained. You learned about the remarkably complicated (and it truly is!) system of sensing and signaling that that the immune cells which occupy this compartment engage in with each other, with the epithelial barrier and with the diverse ecosystem of microbes that live and produce their own families in your gut. You should now also have some appreciation for how genetic mutations in IBD sufferers might contribute to a runaway inflammatory response if, for example, the mutation reduces the activity of the regulatory T cells.

Believe it or not, you are now very close to the frontiers of scientific understanding of these key cellular processes. Working with a system as simple as a fruit fly, scientists continue to make important and exciting new discoveries about the music to which these cells dance. So now it's your turn to step onto the stage. See if you can come up with a new experiment that can uncover additional details in the gut epithelial inflammatory response using the "Smurf

Transition" technique and variables of your own choosing. As you saw at the beginning of this unit, detergent added to the fly's food will disrupt the epithelium and induce a leaky gut. But what else can make the gut leaky? Are there things that help protect it? To get some ideas, you might begin by doing an internet search for foods people eat to reduce inflammation. Thinking about your model and the different kinds of sensors you learned about, what might explain the benefits people get from the remedies they try? Can you replicate this protective effect in a fruit fly? What about the things that can inflame our guts (even those without IBD!)? Why do they cause inflammation? Will they trigger inflammation in a fruit fly as well? How will you set up your experiments to show this?

Planning and setting up fruit fly cultures:
Determine how many fly cultures you'll need and mix the appropriate amount of culture medium according to package instructions. After approximately one week of exposure to experimental treatment, flies will be transferred to fresh medium with 2.5% FD&C Blue Dye and allowed to feed overnight. The next day, flies will be anaesthetized and observed under the microscope to assess gut integrity.

Day 6: The Cell Biologist's Toolkit

How do you measure what a cell does? You may have been wondering as you read the various papers about receptors and signaling and watched the animations just how the heck biologists can observe these things that, for all intents and purposes, must be just about invisible! How indeed! Today your teacher is going to review some of these methods with you so that you can plan an investigation into cellular basis of the effects your treatment may have on the fruit fly.

If you were a scientist conducting these experiments in a university lab, you would need to obtain funding in order to pay for the materials used in your research and for this you would write a grant proposal. To do this, you would need to explain to the committee judging your grant proposal (a) what techniques you plan to use and (b) what you hope these techniques will show.

Typically this would be done as a written proposal but you're going to present your proposal orally and with a slide presentation or poster (at your teacher's discretion). You will have to fit what you want to say into three minutes! Your talk should contain a brief background about the problem of gut epithelial integrity and the inflammatory response and why fruit flies are being used to study the disease process in humans. You should also demonstrate an understanding of the signal transduction and response pathways you've been modeling and how you think they may relate to the smurf transition in fruit flies. With this foundation established, you must explain which techniques you plan to employ and why you selected them. Your classmates will act as the committee and they will have up to three minutes to ask question when you are done. You will, in turn, have up to three minutes to answer their questions.

Appendix

Crash Course Video Homework: The Lymphatic and Immune Systems

Name:_____Period:_____

Date:_____

Lymphatic System (https://www.youtube.com/watch?v=I7orwMqTQ5I)

1. What are the primary roles of the lymphatic system?

2. What are the three main parts of the lymphatic system?

3. What do the lymph nodes and lymphatic glands do?

4. How does lymph move through the lymphatic system?

5. What can happen in a lymph node if an invader arrives there?

6. What are MALTS, where are they found and what do they do?

7. What function does the appendix have?

Immune System part 1 (https://www.youtube.com/watch?v=GIJK3dwCWCw)

1. How does the immune system differ from other organ systems?

2. What is the first line of defense in your immune system?

3. What are some of the innate defenses you have in your mucosal tissues?

4. What's the 2nd line of innate defense, what are some of the cells it has and what can they do?

5. Why is MHC 1 important in innate immunity?

6. What's the inflammatory response? What are the jobs of the temperature changes and histamines?

7. What will cause cells to release pyrogens and what do they do?

8. What if the pyrogenic response doesn't do the job?

Immune System part 2 (https://www.youtube.com/watch?v=2DFN4IBZ3rI)

1. What makes the adaptive or acquired immune system different than the innate defenses?

2. What are the two components of the adaptive immune system and what do they do?

3. How do vaccinations work?

4. Where do the B-cells mature and what does this entail? What do they produce on their surface and where do they go after they mature?

5. What activates the B-cells and what will they do as a result?

6. How do plasma cells and memory cells differ in terms of function?

7. Antibodies can't directly kill invaders but what can they do?

8. What's the secondary immune response and how do vaccines activate it?

9. Why do we need new vaccinations to be protected from some pathogens?

10. What is passive immunity and how does it work?

11. Can B-cells target a pathogen that gets inside of our cells?

Immune System part 3 (https://www.youtube.com/watch?v=rd2cf5hValM)

1. What is the cellular mediated response and what has to happen before it will be triggered?

2. What cells are the key players in this response?

3. What are antigen presenting cells?

4. What is MHC 1, what kinds of cells are they found on, and what are their counterparts on the "professional antigen presenting cells?

5. Where do the T-Cells mature, what kinds are there and what are their roles?

6. What do the T-cells do when activated that resembles the activated B-Cells and what do they do that's different?

7. What does the cytotoxic T-cell do?

8. Why are helper T-cells so important?

9. Why do B-cells wait to get the go ahead from the helper T-cells?

10. What does this check-and-balance system usually prevent?

11. What role do the regulatory T-cells carry out and what are some of the diseases that occur if this regulation doesn't work?

Reading Guide for "Gut Microbiome: The Peacekeepers"
Name: _____Period:_____ Date:_____

1. What did Harry Sokol find in his Crohn's disease patients and what question did it inspire him to ask?

2. His follow up studies seem to have supported his thinking but by what mechanism does this protection seem to work?

3. About a year after this article was published, the estimate of how many bacterial cells an average human body harbors was revised down substantially. Do you think this change in our understanding of microbial abundance will have a big impact on the way we think about their influence on our biology?

4. Perhaps more interesting than sheer numbers may be the species diversity reflected in our gut microbes. In this respect, what is meant by the suggestion that our immune system is "farming" these bacteria? And how might they farm us?

5. "Clostridial cluster" is a descriptor of the phylogenetic relationship among these bacteria. Given that C. difficile (often referred to by researchers as just "C. diff") is a pathogenic species, it is tempting to hypothesize that it either evolved from a commensal ancestor or that their common ancestor was pathogenic and that an early divergence may have given rise to a pathogenic line and a commensal line. How could you test these hypotheses?

6. What does C. diff do in the host gut? What do its commensal relatives do?

7. Besides simply showing that higher numbers of the same bacterium are associated with lower incidence of post-operative inflammatory bowel in both Japan and the US, what else does the author point to as encouraging in the outcome of the US and Japan studies?

8. In a paper he wrote in 1989 (“Hay fever, hygiene and household size”), epidemiologist David Strachen proposed that the rising incidence of allergies might be related to the maintenance of more germ-free environments owing to more careful hygiene and growing up with fewer siblings. What other changes in the human environment and behavioral repertoire can be added to the “hygiene hypothesis” as explanations for our current increase in incidence of allergies?

9. What changes to our microbiome may have been the most problematic for us and how does the author think this may contribute to disease?

10. What do the “peace-keeping microbes” scavenge from our diet and what do the cells in our gut get from this? When this bacterial food source is in short supply, what can the microbes turn to as a source of nutrition?

11. What important change did research using germ-free mice show is likely to occur in us in the absence of a healthy microbiome and how does this impact our allergic reactions?

12. What did Kenya Honda discover about the relative importance of gut microbes in the maintenance of a healthy immune system and what is Vedanta Biosciences doing with this information?

13. What is the evidence that a range of disease pathologies are the result of administration of antibiotics early in life and why does it appear that these diseases may have developed?

14. Why does Lara Hooper say the mucus in our gut provides both a “carrot” and a “stick” to the bacteria that live there? What are the cells that produce this mucus and how do they respond to the presence of clostridial clusters?

15. How might certain species of microbes in our gut influence the environment in a way that benefits other friendly microbes?

16. What did researchers find when they studied patients with and without inflammatory bowel disease who possessed the same NOD2 mutation that is frequently associated with the disease?

17. What does the fact that not all of these individuals had inflammatory bowel combined with the much lower incidence of the disease in adults who grew up on farms suggest about the way we may want to think about managing this disease in the future?

18. Throughout this article we've seen references to ecological concepts and one of the most talked about concepts among modern ecologists is the importance of high species diversity in maintaining robust, stable ecosystems. What do the comparisons of gut microbiomes in North Americans to those of rural residents in Africa and South America imply about the bacterial ecosystems of our guts with respect to this species richness-ecosystem resilience principle?

19. What are the products of bacterial fermentation of soluble fiber in our guts that are thought to contribute to a more stable immune phenotype and how do scientists think this may work? What else does Mazmanian think may be at play?

20. What do studies of mice and people show with respect to the challenge of restoring a healthy microbiota with changes in diet?

21. What did researchers observe when they transplanted microbes from healthy donors into patients with metabolic syndrome?

22. Why is Sonnenburg considering a strategy of exterminating the existing microbiome in some patients, administering immune suppressants and then introducing a favorable microbiome? What risks might this entail?

23. How does McFall-Gnai think animal gut evolution may relate to competition in an environment of scarcity and how did the symbiosis support a mobile lifestyle if she's right?

24. How might our mucus secretion have evolved to keep the population of microbes in check?

Quiz for "Gut Microbiome: The Peacekeepers"

Name: _____Period:_____ Date:_____

1. What did Harry Sokol find in his Crohn's disease patients?
    a. mutations in the patient DNA at loci that code for gut immune cell characteristics
    b. a thinner, less viscous mucus lining on the gut epithelium
    c. one of the species of bacteria whose relative abundance seemed low
    d. the majority of their intestines were necrotic and had to be removed to prevent sepsis

2. Many of the researchers studying the interaction between our gut microbiomes and inflammatory conditions
    a. are starting businesses to communicate the results of their "translational research" to the reading public
    b. became interested in this work because they or someone they love suffers from it
    c. are recommending diets higher in fat and sugars and lower in soluble fiber content to achieve a healthy microbiome
    d. are encouraged by results that suggest simple, inexpensive readjustments of the microbiome may bring relief even to patients with genes that make them uniquely vulnerable

3. Research suggests a highly structured co-evolutionary relationship between the gut epithelia and the microbiome. In fact, it appears that one of the strategies we evolved to help the "peace-keeping microbes" is
    a. epithelial secretions of a sustaining food supply for these microbes should our diet be temporarily deficient of what they require
    b. the ability to sacrifice our own cells as a food source when we're not taking in food
    c. the production of chemicals that push the microbes into dormancy so they can stick around until more dietary fiber enters the food stream
    d. a highly acidic small intestine which is favorable to them but lethal to their competitors

4. While researchers continue to catalog mutations that seem to influence susceptibility to gut inflammatory diseases, some have been studied more than others and one of these better understood mutations that was discussed in this article is
    a. TLR20, a nuclear receptor that activates transcription factors for natural antibiotic proteins
    b. a NOD2 mutation which seems to influence susceptibility to gut inflammation at least partly by impacting the composition of the patients' microbiome
    c. one that causes cystic fibrosis because this mutation changes mucus characteristics in a way that slows the diffusion of the antimicrobial peptides through the mucus layer
    d. one that governs expression of the genes that produce food for the microbial community, overfeeding the bad microbes by loading the gut with simple sugars

5. Many students are surprised to learn that our gut lumen is an anaerobic environment. In fact,
    a. some pockets in our gut have been found to support a highly aerobic environment when we are experiencing gut inflammation and the reactive oxygen species (ROS) that this generates culls problematic microbes from the mix, restoring our health
    b. one of the ways our cells seem to recognize which microbes are about is by detecting the short chain fatty acids that the "good" microbes produce through fermentation of soluble fibers
    c. more and more researchers are of the opinion that aerobic respiration by cells deep within our bodies is the exception rather than the rule because only our epithelial cells are directly exposed to the oxygen-rich atmosphere
    d. the bacteria that are good for us are all obligate anaerobes and those that cause inflammatory conditions are facultative aerobes that go rogue when we eat fast and swallow too much air

Reading guide for: Toll-like receptor signaling in the intestinal epithelium: how bacterial recognition shapes intestinal function
Name: _____Period:_____
Date:_____

Directions: Your teacher will assign you to read one or more sections of this paper. You must understand it well enough to explain it to your peers. There will be some terms and acronyms you may not be familiar with so a glossary has been created to help you as you go. You will also want to make sure you understand how the figures in the paper illustrate the concepts, structures and processes you're reading about because you'll need to use these figures to teach your part of the paper to your classmates. You may find it helpful to redraw the figures for yourself as you read and discuss your part of the paper with your partner.

<u>Good fences make good neighbors</u>

1. What are the cell types that make up the gut epithelium and what does each type do?

2. Where are the stromal cells found, what cell types are found here and what do they do?

3. What are intraepithelial lymphocytes and dendritic cells and why do you think they're not considered to be epithelial cells?

4. How might activity of the smooth muscle layer underlying the lamina propria correlate with the presence of pathogens in the gut lumen (and what *is* the lumen, anyway?)?

5. In the past, what did scientists think the gut epithelium does for us and how has this view changed? (Cite examples from the paper.)

6. The paper you're reading is a "review" that summarizes the findings of many separate "studies." What's the difference between these two kinds of papers?

7. What is the author's purpose in writing this review? (Notice she's using first person here. Her former science teachers might not realize that IT'S OKAY TO DO THIS.)

8. What are PAMPS and how is the way the author intends to write about them different from the way scientists have traditionally thought of them?

9. What's the difference between commensal and pathogenic flora?

10. What effects are TLRs known to have in cells *in general* that the author says are crucial aspects of gut homeostasis?

11. So in addition to their role in maintaining a healthy gut, TLR signals appear to pose hazards through chronic inflammation like we're learning about in IBD pathology. What other disease state is the author interested in learning more about treating as a result of an improved understanding of TLR signaling and gut inflammation?

Sensing bacteria by the intestinal epithelium

12. Pause for a moment to examine figure 2 before you begin reading this section. Look at all the different types of TLRs found in the gut epithelial cells and the differences in the way they're distributed. What do you think the significance of this diversity may be?

13. What does the author mean when she says the IECs are functionally and structurally polarized and how does she claim that this polarity is maintained?

14. What function did this polarity in structure combined with the mucus layer on the epithelium evolve to support and what evidence does the author cite to point out that it "leaks" in healthy individuals but even more so in people with non-functioning TLRs?

15. What would detection of PAMPs by basolateral TLRs be an indication of?

16. The function most biologists from your teacher's generation or before would remember learning that TLRs are critical for detecting invaders and warning the immune system so it could mount an appropriate response. What would these biologists probably find surprising about the expanded role of TLRs in the gut lumen?

TLR expression in the intestine

17. What did scientists set out to find out with regard to commensal bacteria and TLR expression in IELs?

18. What techniques did they have to use and why?

19. What did they find?

20. Sometimes cell biologists will measure gene expression both in terms of the relative amount of mRNA expression and the relative amount of the protein or polypeptide corresponding to the mRNA that may be present in the cell. Why measure both?

21. Aside from determining how abundantly a gene's expression is in terms of the amount of mRNA or protein, what else would a biologist want to know about the gene's product(s)?

22. How does having an inflammatory bowel condition appear to affect TLR expression?

23. Cytokines are often thought of as pro-inflammatory and TLRs as being "for" alerting our immune systems that pathogens are about. But what alternative role may some of the cytokines and some of the TLRs have in gut homeostasis?

24. Which cytokines and which TLRs may be helping keep the calm in healthy guts?

25. What are germ-free mice and what did studies comparing their IECs to those of guts with conventional flora show?

26. What kind of information can investigators get from using immunohistochemistry instead of simply studying gut scrapings?

<u>TLR expression by cell lineage in the intestine</u>

27. So far we've seen that there are multiple TLRs expressed in IECs and their diversity in kind may reflect a similar diversity in functions, with some promoting and some suppressing an inflammatory response. But we also saw that there is diversity in the types of cells that make up the gut epithelium. What do the enteroendocrine cells seem to do when pathogens enter the gut and what role might the TLRs eventually be found to play in this response?

28. Why might localization of gene expression to the crypts indicate activity of the enteroendocrine cells?

29. What is the difference between *in vivo* and *in vitro* and why might results from an *in vitro* study differ from those of an *in vivo* study?

30. How did enteroendocrine cells respond to PAMPs *in vitro*?

31. How does location on the epithelium interact with specificity of TLR expression and the localization of these TLRs on individual cells?

Spatially restricted TLR expression by polarized IECs

32. What's the difference between an apical and a basolateral localization of a TLR on an IEC?

33. Considering how the environment on these two surfaces may differ, why might it be just as important to understand this aspect of specificity as it is to understand which specific cell types express which genes and why?

34. What are T84 and Caco-2 cells and why might researchers choose them for studies in which they want to examine polarized responses to PAMPs *in vitro*?

35. What general statement, if any, might be made with regard to the studies of TLR expression in polarized cell lines *in vitro* and what does it suggest about the possibility of polarized expression *in vivo*?

36. Why should expression of PAMP detecting receptors differ between the lumen of the intestine and the basolateral surface of the same cells?

37. According to comparisons with germ-free mice, how does the presence of commensal bacteria seem to impact expression of TLR genes and their proteins?

38. How do cell identity (e.g. immune vs. non immune cells) and cell surface (in the case of polarized cells) differ with respect to the response to CpG ODN (a PAMP)?

39. What might be the significance of differences in signal transduction pathway activation or inactivation by cell type and surface?

40. What might it mean that results from studies of human cell TLR9 responses differed from the results of mouse studies?

41. Why would a study showing higher quantities of a specific mRNA not show a similarly elevated level of its protein?

42. Why, according to the author, should inflammatory signals and cell responses be restricted to the basolateral surface of the mucosal epithelium and what does the presence of PAMPs in this location indicate?

Intracellular expression of TLR4 by IECs

43. Where have some investigators found TLR4 proteins and why was this surprising?

44. If this result is successfully replicated in subsequent studies, what might it suggest about the function of isolation detectors on the cell's interior and bringing the PAMPS in to them?

45. What were the limitations of these studies and why should that lead us to be cautious about their interpretation?

Negative regulators of TLR signaling in IECs

46. Why should IECs express genes whose products police TLR signals and responses?

47. What is TOLLIP and how does it work?

48. How does Tollip expression differ in patients with IBD?

49. What is SIGIRR and how does it appear to work?

50. What is PPAR-gamma and how does it work?

51. Why would low levels of MD2 suppress inflammatory responses?

Sensing intestinal injury through TLRs

52. Where on the intestinal epithelium are the crypts and how do the cells here give rise to the rest of the epithelial cells? What are WNT, Notch and Hedgehog factors and what role might they play in this differentiation?

53. What are the components of the stem cell niche that lie beneath the crypts?

54. How do germ-free mice compare to those with a conventional flora in terms of the rate of cell proliferation in the crypts and of what interest are the TLRs with respect to this difference?

55. What did investigators hope studying mice that either lacked functional TLRs or MyD88 would show them?

56. How does proliferation and barrier integrity in mice with no TLR signaling compare to normal mice under normal (non-injury and in the presence of conventional flora) conditions?

57. Why, according to the author, might this be the case in light of other evidence that IECs sense bacteria through this signaling system?

58. What other signaling system does she think might explain this?

59. What is DSS used for in gut epithelia experiments and what does treatment of mice with deficiencies in TLR signaling OR with a gut flora disrupted by antibiotics reveal about the role of the bacteria and this signaling system under non-steady state conditions?

60. In addition to using mice with genetic deficiencies in TLR or its adaptor MyD88, researchers used normal mice treated with antibodies to the TLR4 and LPS agonists. What might these experiments be able to show that experiments using the genetic mutants (“knockouts”) wouldn’t show?

61. What are the events that follow activation of a TLR4 receptor on the membrane of an individual cell and which may explain how its activation promotes cell proliferation in DSS injury?

62. TLR4 activation of IECs also appears to lead to recruitment of macrophages to the injury site and mesenchymal stromal cells to the stem crypt. What role do they appear to play in repair when they arrive and what molecules seem to be needed both for their relocation to occur and to trigger their secretion of proliferation-inducing factors?

63. What was the design of the experiments that were used to show that COX2 and MyD88 were critical to inducing the signals for cell proliferation response in injury conditions?

64. What are the ligands for TLR2 and 3 and how does their activation appear to modulate epithelial injury?

65. What ligand have researchers found to induce protection of the epithelium through the TLR9 receptor, which cells are activated by this binding and how do they produce their protective effects?

<u>Radiation induced injury</u>

66. Patients who are undergoing radiation treatment for tumors often experience hair loss, compromised immunity and gut irritation due to the toxicity of the treatments to specific cell populations and their therapy must be suspended while these tissues recover. What makes all of these cells so susceptible to radiation damage?

67. How might the TLR signaling research that was reviewed in this paper (and follow up studies) be applied to helping physicians lengthen the amount of time patients can endure chemotherapy treatments without gut injury?

68. How did LPS treatment of mice prior to radiation treatment protect them?

69. What effect did administration of flagellin prior to radiation have in mice and monkeys and what are the components of the signaling pathway?

70. In addition to direct oral or systemic administration of PAMPs, how might physicians eventually be able to activate protection of the gut in radiation patients?

Necrotizing enterocolitis

71. What are the effects of NEC in infants and, according to studies in mice, what role do TLR2, TLR4 and TLR9 appear to play?

72. With respect to NEC prevention and treatment, what kinds of protocols appear promising as a result of increasing knowledge about TLR signaling and its role in both the injury and protection of the gut epithelium?

TLRs regulate barrier function

73. What are tight junctions composed of and what is their function?

74. What effect did the introduction of a commensal bacterial species have on the expression of genes for these tight junctions in the gut epithelia of germ-free mice?

75. By what mechanisms does TLR2 appear to promote this effect?

76. How did TLR2 activation affect resistance to colitis induced by pathogenic bacteria, promote barrier integrity in cell culture and recovery from DSS-induced colitis?

77. Why do you think investigators chose MyD88 and TRIF deficient mice to demonstrate that epithelia in the “steady state” (not exposed to inflammatory conditions) don’t require TLR signaling to maintain barrier function?

Antimicrobial peptides and leptins

78. What do defensins do to bacteria and which cells can produce them?

79. Paneth cells produce most of the other antimicrobial molecules but recent studies have also shown that goblet cells are a source of angiogenin. This molecule has recently been found to cause agglutination of gram negative bacteria. What does this mean and how would it interfere with the success of the bacteria?

80. REG3gamma attaches to gram positive bacteria by recognizing their petidoglycans, oligomerizes and forms a pore. Look this up and illustrate what it looks like.

81. What are the PAMPs that were used to trigger alpha-defensin secretion in isolated Paneth cells?

82. What does normal alpha-defensin synthesis by MyD88 deficient mice and its reduced synthesis by NOD2 deficient mice or TLR9 deficient mice suggest about the regulation of this antimicrobial compound by cells?

83. How does introducing bacteria to germ-free mice affect REG3gamma production by Paneth cells and what did the investigators do in the experiments described to demonstrate that this involved TLR signaling and that probably many TLRs were involved?

84. Where are the mesenteric lymph nodes and what would an increase in bacteria detected here indicate about the gut epithelial barrier?

85. What effect did administration of broad spectrum antibiotics have on antimicrobial peptide secretion and what concern does the author therefore express about their use?

<u>Shaping the mucosal immune response</u>

86. The author states that studies have shown IEC-microbe interaction shapes innate and adaptive immune responses in the lamina propria but she does not say how this epithelial interaction is transferred into the compartment beneath the epithelium. Given what you've seen so far what are some of the mechanisms you would propose?

87. Since IgA molecules are antibodies, they are the products of B-cells. What are the different IgA types and how do they differ?

88. Why might the IECs have evolved the ability to trigger a switch between IgA types? What does TLR activation do that may explain it?

89. What does the author mean when she writes that the mouse model had a constitutively active form of TLR4 in the intestinal epithelium? What was observed in this mouse model?

90. How do dendritic cells sample the gut microbiome and how does TLR signaling appear to affect this?

91. How can experiments that involve localized deletion of NFκB or TAK1 functioning in IECs indicate the importance of TLR activity in barrier function?

92. If bacteria can induce IECs to produce the cytokines TSLP, TGF-beta and IL-25 and each of these in turn has the ability to modulate the level of response by the anti-pathogenic helper T cells, T_H2 and T_H17, and decrease dendritic cell vigilance, what kind of distinction must these IECs also be helping the immune system to make among the species in the gut lumen?

Trefoil factor

93. How do the cells on the margins of an epithelial lesion change to promote healing (restitution) and what role does TFF3 in the mucus layer that the goblet cells produce seem to play in this response?

94. What TLR2 ligand appears to upregulate the expression of TFF3 and what might this mean for treatments of some types of colitis?

TLR signaling and colorectal cancer

95. Throughout this paper we learn about the cell-cell interactions that are involved in severing and repairing connections between cells, triggering cell death and promoting cell division and migration. In what ways do each of these processes relate to the development and/or control of tumors?

96. Given that APC is a protein that plays a critical role in regulating cell responses along each of these lines, it's not surprising that mutations in this gene would be implicated in colon cancers. In fact, its wild-type form is considered to be an anti-cancer gene. In experiments comparing tumors in APC-knockout mice grown in either germ-free conditions or with conventional flora, how did the composition of the chow represent a confounding variable and what might it imply about TLR involvement in APC pathways, and why did using MYD88 mutant mice suggest TLR involvement?

97. What is a bone marrow chimera and why would an experiment using them help to eliminate the possibility that TLR activity in hematopoietic cells is involved directly in tumorigenesis (neoplasia)?

98. What role did SIGIRR play in tumor regulation and what would this have to do with TLR activity?

99. How do cytotoxic T cells and NK cells attack tumor cells?

100. Why should the final paragraph give pause to anyone thinking that nonspecific downregulation or upregulation of TLR activity would be a promising approach to treating colon cancer? What might be a better approach given what is known so far?

Glossary for: Toll-like receptor signaling in the intestinal epithelium: how bacterial recognition shapes intestinal function

Absorptive enterocytes – IECs that make up the majority of the intestinal epithelium and play a primary role in nutrient absorption

Amphiregulin – an EGF that promotes proliferation of gut epithelial cells

APRIL – a proliferation-inducing ligand; a cytokine from the TNF superfamily; a B-cell stimulating factor that promotes B-cell survival and class switching

Basement membrane – an extracellular mesh made up of protein fibers which provides a scaffold to which the cells of a tissue are anchored

B-cells – these are cells of the adaptive immune system which produce antibodies that can bind with molecules projecting from the surfaces of non-self cells.

Beta-catenin – TLR4 activation in NEC decreases cell renewal through this pathway in isolated small intestine IECs; this protein is also a subunit of the cadherin cell junction complexes and intracellular transducer of WNT signaling pathway; Drosophila has a homolog called armadillo.

Beta-cellulin – an EFGR ligand that plays a role in gut epithelial cell proliferation, differentiation and migration

CCL20 – this cytokine is important for recruiting lymphocytes and dendritic cells into the lamina propria; it is a chemoattractant for these cells and acts as a ligand for their CCR6 receptors.

CCR6 – chemokine receptor 6; a receptor on lymphocytes and dendritic cells that triggers cell migration along a gradient of the chemokine CCL20; chemokine gradients tell immune cells where they are needed.

Commensal microbiota – bacteria and other single-celled organisms that appear to be able to live and reproduce within the microscopic ecosystem that lines our epithelium (including our skin); some are known to have a mutualistic relationship with our bodies but others may neither harm or help us (somewhat like a bird's nest on a tree branch).

COX2 – cyclooxygenase 2; one of a set of enzymes involved in synthesis of inflammatory lipid hormones known as prostaglandins; aspirin and ibuprofen are two nonsteroidal inflammation drugs (NSAIDS) that act as inhibitors of these enzymes.

CpG-ODNs – short, single stranded, synthetically produced nucleotide chains with a much higher frequency of cytosine-guanine repeats than is typically found in vertebrates; researchers use them to trigger immune responses based on studies that found these pairs to be common in

microbes, they are therefore a type of PAMP. TLR9 is the PRR most often associated with these PAMPs in the literature.

Crypts – the lowest points between the villi; this is an important zone of cell proliferation in the gut epithelium with new cells being produced at a pretty high rate in a healthy gut and migrating toward the apex of the villi to replace dying cells as though on a conveyor belt.

Cytokines – a class of small proteins and glycoproteins that serve as ligands for the receptors on a range of different types of cells in the innate and adaptive immune systems. Each of these cells has a very specific set of receptors for detecting a unique set of cytokines allowing their responses to these cytokines to be very specific as well. Depending on the unique relationship between cytokine and its "target" cells, the recipient cells may respond by producing cytokines that will activate other immune cells, directly initiate a destructive sequence of events that will promote inflammation or prevent/suppress inflammation. These cytokines may be produced by many different kinds of immune cells but also by somatic cells that are not considered part of the immune system.

CXCL2 – CXC-chemokine ligand 2 (also called macrophage inflammatory protein 2 or MIP2); a cytokine that was shown in mouse intestinal epithelia to increase neutrophil and lymphocyte recruitment

DSS – dextran sodium sulfate; essentially a detergent used by researchers to induce lesions in the gut epithelium; while still commonly used, this method is criticized by some experts who feel that gut lesions induced in this way may not closely mimic the natural process of inflammation (typically induced biochemically in response to gut lumen microbes or food toxins); some researchers are beginning to adopt protocols that stimulate inflammation by introducing actual pathogens.

Endosome – these are vacuoles found within the cytoplasm which are formed when the cell takes in material by endocytosis; they serve an important role in sorting and processing material and, as materials are sorted and the endosomes "mature" their contents can be processed. In some cells, specific TLRs are transferred from the golgi to these compartments and are not expressed on the cell surface.

Enteroendocrine cells – IECs that produce hormones such as serotonin, vasoactive intestinal peptide and somatostatin, which regulate secretion of fluids and electrolytes, motility, blood flow and food intake; also responsive to PAMPS such as CpG-ODNs, which triggers secretion of cholecystokinin – a hormone that causes the gall bladder and small intestine to contract

EGF – epidermal growth factor; this is a hormone that stimulates epithelial cell growth, proliferation and differentiation.

EGFR – epidermal growth factor receptor; expressed by IECs

EP - prostaglandin E receptor; these play an important role in binding cell proliferation signals and triggering production of EGFR ligands.

Epiregulin – an EFGR ligand that plays a role in gut epithelial cell proliferation, differentiation and migration

FAE – follicle-associated epithelium – this is a subset of the gut epithelium that overlies the Peyer's patches or GALTs (gut-associated lymphoid tissues); dendritic cells which reside here are able to reach between these cells and sample the gut flora. By doing this, they can census the populations of commensal and pathogenic bacteria and trigger an inflammatory response if pathogen numbers are increasing by making and secreting cytokines to signal the various other immune cells (lymphocytes) that also reside here.

Flagellin – a structural protein used in bacterial flagella. Some IECs have the ability to detect and release cytokines in response to this protein.

Flora – another name for the collection of microbes that live in and on our bodies.

GALT – gut-associated lymphoid tissue; there are lymph nodes (Peyer's patches) scattered among the villi in your gut epithelium and they play a critical role in epithelium homeostasis. A healthy gut will generally have thousands of species of commensal microbes that are recognized as harmless by the lymphocytes that reside here. An infection by pathogenic microbes will be detected at these Peyer's patches by the ever-vigilant lymphocytes that are constantly monitoring them. Activated lymphocytes will migrate through the lymph in the lamina propria to the surrounding villi to battle invading pathogens.

Goblet cells – IECs that produce the mucus layer which lines the gut epithelium

G-CSF – granulocyte colony-stimulating factor; has a modulatory effect or monoyctes, macrophages, dendritic cells and T-cells; but used to trigger cell division in bone marrow

HB-EGF – heparin binding epidermal growth factor; an EFGR ligand that plays a role in gut epithelial cell proliferation, differentiation and migration

Hedgehog – Hedgehog proteins are one of the most studied groups (they come in many related forms) of signal proteins because they play important roles in so many signaling pathways and mutations affecting their function play a role in the development and expression of so many diseases. They're known to have important effects on the growth, survival, and fates of cells and these effects depend on cell type, tissue type, dosage and timing of expression. Indian hedgehog is known to play a role in negative feedback inhibition of crypt stem cell proliferation and a drop in its concentration stimulates cell proliferation as well as immune system activity in the lamina propria.

IECs – intraepithelial cells; the single layer of cells that comprise the gut epithelium; this list includes four types: absorptive enterocytes, Paneth cells, goblet cells, enteroendocrine cells.

IFN – interferon; named for their ability to interfere with viral replication, this family of cytokines can be expressed by almost all of our cells when invaded by viruses, bacteria or other pathogens as they enhance the sensitivity of neighboring cells to viruses by enhancing their expression of the MHC molecules that these cells can use to present antigens to the immune system; they also recruit Natural Killer cells and macrophages, which are both innate immune cells that can destroy and recycle the components of infected cells; macrophages can activate the adaptive immune system.

IFN-gamma – interferon gamma; this is a cytokine that can be produced by a number of different kinds of cells throughout the body where it may play a role in inhibiting viral replication, trigger inflammation or modulate an inflammatory response; of course which outcome is activated depends on the kind of threat detected (and whether or not there really IS a threat when it's acting in an anti-inflammatory manner) and what kind of response the local community of target cells is designed to mount; in the gut it's generally pro-inflammatory.

IgA – immunoglobulin A; this is an antibody that B-cells in the lamina propria produce; there are different subtypes of this antibody that differ in their specificities and probably also in the roles they play in regulating the relative abundance of different microbe species in the gut; considered an essential feature of mucosal immunity to infection and produced in large amounts daily; IgA1 is T-cell dependent and antigen specific but IgA2 is not and has longer persistence due to its resistance to bacterial proteases; switching between the 2 is triggered by IECs through TLR activation.

IκB-alpha – this protein acts as an inhibitor of NF-κB-alpha.

IL-# - interleukin; this is a class of cytokines which are produced by immune cells to control an immune reaction; some tend to be pro-inflammatory while others are anti-inflammatory; unfortunately, the numbering system used to identify them offers little help in trying to figure out which effect they predominantly have because these numbers are assigned as new ILs are discovered – which is not an infrequent occurrence!

IL1-beta – this cytokine is also known as leukocytic pyrogen due to its inflammation promoting properties; it's known to promote cell proliferation (for which it's referred to as a mitogen), differentiation and apoptosis.

IL-1R – IL1 receptor; this receptor comes in two forms, one which promotes inflammatory responses and the other appears to suppress them by competing to bind the signal.

IL-4 – this interleukin is derived from T-helper-2 (T_H2) cells and it decreases the sensitivity of IECs to LPS (a pro-inflammatory trigger from bacteria), possibly by causing these cells to decrease the quantity of TLR-4 molecules (LPS receptors) they make.

IL-6 – stimulates IECs to proliferate when an injury occurs and may inhibit apoptosis

IL-8 – a cytokine that acts as a chemokine which is especially attractive to neutrophils whose phagocytic activity is upregulated by this cytokine; this cytokine is a ligand for the receptors CXCR1 and 2.

IL-13 - this interleukin is derived from T-helper-2 (T_H2) cells and it decreases the sensitivity of IECs to LPS (a pro-inflammatory trigger from bacteria), possibly by causing these cells to decrease the quantity of TLR-4 molecules (LPS receptors) they make.

IL-33R – interleukin 33 receptor; IL33 is a member of the IR1 cytokine superfamily and its expression is upregulated by epithelial cells under inflammatory conditions and alerts the immune system to necrosis and tissue damage; the receptor is highly expressed in cells of the innate immune system and T_H2 cells; effects have been shown to be inflammatory in some tissues and anti-inflammatory in others.

IRAK – interleukin-1 receptor-associated kinase 1; this is a kinase that, when IL1 receptor is activated, activates the NFκB pathway.

JNK – c-Jun N-terminal Kinase; kinases are intracellular enzymes that transfer phosphates from high energy molecules such as ATP to a wide range of molecules with the result of changing their activity – often turning them on or off; c-Jun is the name for the substrate that this kinase phosphorylates and the phosphorylation occurs at the N-terminus or the end with the unbound amino group (the other end of a protein or peptide is the C-terminus and it has a carboxyl end that's not engaged in a peptide bond); JNK is a key component of many stress response pathways, apoptosis, T-cell differentiation, and inflammatory responses; it's a member of the MAP kinase family.

Lamina propria – a space beneath the epithelial cells of the gut where cells of the innate (e.g. dendritic cells, macrophages, monocytes, neutrophils) and adaptive (B-cells and several kinds of T-cells) immune systems localize (following inflammation signals) or lurk waiting to intercept pathogens that penetrate the epithelium or to receive instructions from other cells to either trigger an inflammatory response or prevent one

Lectin – antimicrobial products that many of our cells have the ability to make but in the gut epithelium they're mostly derived from Paneth cells; includes REG3 gamma and beta, CRP-ductin and resistin-like molecule beta

Lipoteichoic acid – a surface associated adhesion molecule released from gram positive bacteria when they are degraded

LPS – lipopolysaccharide; one of the most well known PAMPS; a molecule derived from bacteria that our cells use to detect the presence of pathogenic bacteria and which researchers commonly use in experiments when they wish to trigger an inflammatory response

MD2 – myeloid differentiation-2 protein; this molecule forms a dimer with TLR4 and is necessary for TLR4 to respond to its ligand, LPS

Mesenchymal stromal cells – these are a kind of stem cell that reside in the lamina propria; are not very lineage specific and therefore may be able to differentiate into a wider range of adult cells but in the gut epithelium they seem to secrete factors that induce proliferation of IECs; they may also modulate the inflammatory response.

MyD88 – myeloid differentiation primary-response protein 88; MyD88 is an intracellular adaptor protein that plays an important role in TLR signal transduction. Adaptor proteins don't usually have any enzymatic activity but aid in the formation of associations between other proteins in a signal transduction pathway, enabling TLR to activate the NF-κB transcription factor. In the case of TLR2 and TLR4 activation, MyD88 recruits the IRAK pathway. MyD88 knockout mice were found to be susceptible to bacterial infection but humans don't seem to be as immunocompromised when they lack functional MyD88.

Myofibrolasts – these cells are located beneath the crypts and, along with small blood vessels also located here, are considered part of the "stem cell niche"

NEC – necrotizing enterocolitis; a disease common in prematurely born infants; rapid spread and spread of pathogenic bacteria triggers inflammatory response that destroys a region of the gut

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B-cells; an intracellular protein produced by almost all cells and used in a variety of different signal transduction pathways and therefore associated with a number of diseases and disorders; it's a transcription factor that plays an important role in transducing cytokine signals.

NOD – Nucleotide-binding oligomerization domain; this is an intracellular receptor that appears to have evolved to detect the bacteria that have breached the cell and are therefore no longer visible to cell surface receptors of other cells; they can be relatively specific in terms of which kinds of gene transcription pathways they trigger; responses to NOD activation include cytokine signaling (as, for instance, to recruit natural killer or NK cells) and autophagy/apoptosis; NOD2 is known to play an important role in gut homeostasis by responding to peptidoglycan released by bacteria in the gut, triggering the kinase receptor-interacting protein 2 (RIP2) which leads to activation of NFκB; activation of this system generates antimicrobial peptides (esp. alpha defensin) and mucin which help maintain a safe distance distance between the epithelium and the microbiota; Broadly, variation in NOD activation can produce a range of responses from pro-inflammatory to anti-inflammatory.

Notch – This receptor comes in a few different variants which are found in cells throughout the body and play important roles in a number of cell processes. Their ligands trigger proteolytic cleavage and release of an intracellular portion which enters the nucleus and modifies gene expression. It's been found to play a critical role in maintaining the "stemness" of stem cells in

the crypts in the gut epithelium. In experiments in which notch was inhibited, this population of cells went into decline and some began differentiating into secretory cells with goblet or paneth characterisitcs prematurely.

PAMP – pathogen associated molecular patterns; this acronym is used to refer to any of a number of molecules that may indicate the presence of a non-self cell; these may be signaling molecules released by such cells, metabolic byproducts or membrane-bound markers and traditionally the idea was that the ability to detect these markers would allow the immune system to mount a protective response to pathogens before they could make us sick but it is also now used to refer to molecular cues from "good" bacteria.

Pam3CSK4 – tripalmitoyl-S-glycerol cysteine-serine$_4$-lysine; a TLR2 ligand that helps protect the epithelium against DSS-induced inflammation

Paneth cells – IECs that produce antimicrobial compounds (such as defensins, angiogenin 4, REG3) and lectins (which can assist cell-cell interactions without involving the immune system)

Peyer's patches – these are typically dome-like structures that are scattered among the villi on the gut epithelium; they're specialized lymph nodes containing many kinds of lymphocytes that constantly monitor the gut flora and respond to changes in microbe populations by triggering inflammatory responses when pathogenic populations spike and activating anti-inflammatory cytokine production in response to commensal species.

PGE2 – prostoglandin E_2; this prostaglandin has a wide range of effects in cells including effects that relate to gut homeostasis; it appears important in mucosal protection, secretion and motility and some IBD patients with mutations in PGE_2 receptors or in proteins from the pathways these receptors control may be unable to benefit from the modulating effects of prostaglandin; it's a potent activator of the Wnt pathway.

Poly I:C – polyinosinic polycytidylic acid; acts through TLR3 signaling pathway to modulate DSS-induced colitis

PPAR-gamma – peroxisome proliferator activated receptor-gamma; this is a nuclear receptor and transcription factor which activates genes known to play an important role in triggering fat uptake and storage; it's expressed mainly in adipose tissues (fat), and colon and macrophage cells; most studies of this receptor relate to glucose regulation in type2 diabetes for its abilities to increase insulin sensitivity; increasingly, this "promiscuous" (because it accepts such a wide range of molecules as ligands) nuclear receptor has gained much attention by researchers interested in its ability to suppress inflammation by suppressing expression of inflammatory cytokines; its ligands include various xenobiotics, vitamins and metabolites so diets that provide an abundance of these molecules may help to prevent inflammatory episodes.

PSCs – pluripotent stem cell-derived mesenchymal stem cells; these cells have the capacity to differentiate into a number of different kinds of cells and also can have a modulatory effect on

immune responses; in the gut epithelium, they appear to be important for signaling cell proliferation under injury conditions.

PRR – pattern recognition receptors; these are receptors in host cells that have evolved to detect unique classes of molecules that are associated with pathogens (PAMPs) and which are able to initiate an immune response when a relatively low threshold concentration of the pathogen-derived ligands is crossed.

REG3 – regenerating islet-derived protein 3; antimicrobial proteins made by paneth cells;

REG3-gamma – specifically binds gram positive bacteria by binding to their surface peptidoglycans

SIGIRR – Single immunoglobulin IL-1R-related molecule/receptor; known to modulate inflammatory responses, epithelial homeostasis in the colon and tumorigenesis; negatively regulates signaling through the receptors IL-1R, IL-33R, TLR4 and TLR9

Stem cell niche – a region at the base of the villi that includes the cells in the crypt of the epithelium, the underlying blood vessels and the myofibroblasts

TFF3 – trefoil factor 3; secreted by goblet cells, plays a role in gut repair after infection, probably influencing migration of proliferating cells from the wound margin to fill the lesion

TGF-alpha – transforming growth factor alpha; an EFGR ligand that plays a role in gut epithelial cell proliferation, differentiation and migration

T_H# cells - T-Helper cells; this is a subset of immune cells of the adaptive immune system that resides in the lamina propria and regulates inflammatory responses by generating anti-inflammatory or pro-inflammatory cytokines; there are several subtypes of these cells and they are numbered, with new numbers being assigned as new subtypes are discovered.

T_H1 – host immunity effectors against intracellular bacteria and protozoa, triggered by IL-12 and IL-2 and their effector, IFN gamma

T_H2 – these are thought to inhibit inflammation in the gut by inhibiting sensitivity of IECs to LPS by decreasing the production of its receptor, TLR4; the interleukins they produce are IL-4, IL-5, IL-6 and IL-10; of these, IL-4 triggers the differentiation of naive helper T-Cells into additional T_H2 and IL-10 is an anti-inflammatory cytokine so increased synthesis of either of these cytokines would suppress inflammation.

T_H17 – important for clearance of microbes at mucosal surface; produce IL-17; signals that cause differentiation of these cells also suppress Tregs (the anti-inflammatory immune modulating cells).

TLR– toll-like receptors; a receptor commonly used to detect molecules derived from bacteria, fungi and other eukaryotic non-self cells and viruses; these molecules bind to the receptor and can trigger a range of responses depending on whether or not our cells associate them with danger.

TLR2 – expressed by IECs in the colon on the brush border of the IECs on the apical surface of the villi in the presence of commensal bacteria but not expressed in guts that were germ-free (without bacteria); this receptor is expressed by Peyer's patch IECs on both the lumen and lamina propria sides; this receptor has been shown to trigger expression of the CYP1A1 gene which produces an enzyme that helps break down certain carcinogenic dietary toxins; upregulated in NEC and positively correlated with degree of mucosal damage; TLR2 activation phosphorylates protein kinase C alpha and delta which leads to reorganization of ZO1 in tight junctions; IECs in culture treated with TLR2 ligands had improved barrier function as evidenced by increased transepithelial resistance; treating DSS-lesioned mice with these ligands decreased apoptosis and reorganized ZO1; during C. rodentium infection, signaling by TLR2 in the lamina propria is detrimental rather than protective.

TLR4 – expression is increased in the presence of inflammatory cytokines; this receptor is a pattern recognition receptor and its ligand is lipopolysaccharide (LPS), a molecule (a common PAMP) associated with potentially harmful bacteria which can trigger an inflammatory response; expression also is increased by IECs in the presence of commensal bacteria in a non-inflammatory environment; highly expressed by IECs in colonic villi on their surfaces exposed to the lumen in active Crohn's disease

TLR5 – expressed by cells on the apical surface of villi and FAE/Peyer's patches; studies suggest the distribution of this receptor is polarized with expression restricted to the surface that interfaces with the lamina propria as its activation was not detected in the presence of its ligand (bacterial flagellin) unless the cell junctions were disturbed, allowing the flagellin access to this surface of the cells; however, another study in mice found this receptor expressed on the lumen/apical surface of cells with commensal flagellin serving as ligand – their activation triggering release of the mouse version of IL-8 (KC).

TLR9 – expressed by IECs in the colon on the brush border of the IECs on the apical/lumen surface of the villi in the presence of commensal bacteria but not expressed in guts that were germ-free (without bacteria); studies show this PRR to be responsive to CpG-ODNs and it's a receptor most typically found in B-cells and limited subsets of dendritic cells; this receptor is expressed by IECs on both the lumen and lamina propria surfaces of IECs in the Peyer's patches; important stimulator of frizzled 5 receptor and, consequently, alpha-defensin production in small intestine

TNF – tumor necrosis factor; a cytokine that is frequently deployed early in an inflammatory response

TOLLIP – Toll-interacting protein; this is a protein that inhibits activity of TLR2 and TLR4 through IL-1R-associated kinases (IRAKs)

TRIF – TIR(toll interleukin receptor)-domain containing adapter-inducing interferon-beta; this is an intracellular domain found in TLRs and acts an adaptor that, when TLR3 and TLR4 are triggered, together with MyD88 activates expression of specific cytokine genes. TRIF appears to provide some specificity to the TLR-MyD88 signaling. Another adaptor that helps MyD88 specify which cytokine genes to express is TIRAP [toll-interleukin 1 receptor (TIR) domain containing adaptor protein] which is used when TLR2 and TLR4 are activated.

Type I interferons – this is a class of interferons that modulate immune system activity through binding with their own class of interferon receptors.

Ubiquitylation – the enzymatically driven attachment of ubiquitin to proteins; Ubiquitin is typically thought of as marking its target for destruction but it may also change its target's activity.

Villi – the gut epithelium is lined by a single layer of IECs whose surface on the lumen-side of the intestine extends tiny projections (brush border) into the intestinal lumen to increase surface area for absorption of nutrients and water. This single layer of cells is itself folded into larger segments with multiple cells that project into the lumen to further increase surface area.

Wnt – this is a protein that cells use to signal themselves or their immediate neighbors and it comes in many different forms, each with distinct receptors from a similarly diverse group called Frizzled which are associated with G-linked proteins and, on the cytoplasmic side, disheveled is a protein in the signal transduction pathway activated by these ligand-receptor interactions. Mutations in these signals, their receptors and the intracellular proteins that compose the pathways to the genes they activate are involved in a number of diseases.

Xenobiotics – these are molecules that are recognized as foreign by a number of the intracellular receptor types known as nuclear receptors; many of the compounds in prescription and nonprescription drugs act as xenobiotics but xenobiotics are also commonly found in plants.

Xenosensors – these are intracellular receptors (referred to as "nuclear receptors") that recognize various types of molecules that are not made by the organism as foreign; their ability to do this owes to the fact that their "xenobiotic" ligands can impact the biology of the organism and over evolutionary time organisms evolved the ability to sense and respond to these xenobiotic compounds; their ligands include various toxins found in food and synthetic compounds such as might be found in medications; much of the work that has been done to understand them has been focused on their role in drug metabolism but they are receiving increased attention from researchers who study gut inflammatory disorders because many of their ligands appear to help reduce risk of inflammation and these ligands may be increased through changes in diet.

ZO1 – zonula occludens; plays an important role in crosslinking tight junction proteins and anchoring them to the actin cytoskeleton

The Cell Biologist's Toolkit:

Techniques for Studying Cell Activities

Bioinformatics

- Repositories of gene data from millions of studies.
- BLAST tool can be used to search for gene homologs in different species.

in situ / in vivo

- *in vitro* - Isolated cells from animal are grown in culture medium to which specific factors may be added and cellular responses detected.
- Whole tissues may be grown in a culture medium in some cases as in gut epithelium where cells show polarity in organization and function.
- *in vivo* – cell behavior studied within intact organism.

Flow cytometry

- Cells suspended in thin stream of solution pass one-by-one through different colored laser beams.
- Side scatter, forward scatter and fluorescence emitted by antibody-linked fluorophores is used to identify and count unique cell types.
- Can be used to detect cell-type specific protein expression in response to *in vivo* treatments.

gene knockout/knockin

- Knockouts - model organisms with specific genes made nonfunctional by mutation; phenotypic comparison to “wild-type” reveal gene function.
- Knockins – model organisms with targeted insertion of specific genes; many transgenic mice being created with human gene variants in order to study the effects of these genes in a living mammalian model.

gene knockdown

- Used to prevent or reduce translation of specific mRNA transcripts into proteins or disable the proteins themselves to determine their function.
- Treatment can be targeted to specific cell types or tissues and/or specific stage in life cycle.
- Minimizes other phenotypic changes that may occur in gene knockouts or knockins.

Fluorescent proteins

- Naturally produced bioluminescent in certain species of jellyfish.
- Genes for these proteins can be introduced downstream from promoters for non-fluorescing proteins in target species.
- Determining when or where gene is expressed is a simple matter of looking for fluorescing cells.
- Can also be used to label specific cell types, so their activity can be observed.

Immunohistochemistry

- Antibodies that can bind specifically to a molecule of interest (e.g. glycoprotein receptor, secondary messenger protein) are linked to a small fluorescent molecule.
- When applied to sectioned tissues, antibodies will bind their targets and, after washing off remaining fluorescent tagged antibodies and exposing to appropriate light source, expression of these targets can be localized to cell populations or specific cellular compartments.

Immunocytochemistry

- Similar to immunohistochemistry in that antibodies with fluorescent tags are designed for their ability to bind specific antigens.
- Different because immunohistochemistry utilizes sectioned tissue and immunocytochemistry is done with living cells.
- Used to identify types of cells that express a gene and in which part of these cells.

ELISA

- ELISA is an acronym for “enzyme-linked immunosorbent assay.”
- A few microliters of each sample added to wells in a multi-sample plate.
- Target binds to well and antibody linked to color reporter binds to target.
- Can be used to find out how much of a specific protein (e.g. an interleukin) is present in a sample.

Northern blotting

- RNA is extracted from cells and separated into distinct bands in electrophoresis gel.
- Bands are transferred to a “blotting membrane” from which specific transcripts can be labeled by complimentary sequences (“probes”) linked to tag that can emit a color.
- May reveal changes in gene transcription that do not show up as changes in translation to protein.

in situ hybridization

- Like Northern Blotting, technique uses color changing molecular label linked to short nucleic acid sequences that are complementary to target mRNA transcript.
- Used in whole tissue, organ or organism (for very small organisms) to localize expression of specific genes.

qPCR

- In “quantitative PCR” RNA is extracted from cells and loaded into multi-well plate.
- Into each well reverse transcriptase, a specific primer, nucleotides and enzymes are added to allow amplification of specific mRNA transcript as cDNA in thermocycler.
- Allows determination of which genes are upregulated or downregulated in specific cell types.

RNA seq

- Uses thermocycler to make multiple partial cDNA copies of all mRNA in a sample so you can search the whole “transcriptome” for changes in gene activation.
- Results should be validated with qPCR.

DNA Deep seq

- Similar to RNA seq, multiple short copies are made of DNA.
- Used to determine whole genome sequence.
- Can be used to identify the species that make up a microflora.